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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>6</sup> :</b> <b>A61K 48/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/19793</b> <b>(43) International Publication Date:</b> 27 July 1995 (27.07.95)
<b>(21) International Application Number:</b> PCT/US95/00817 <b>(22) International Filing Date:</b> 20 January 1995 (20.01.95) <b>(30) Priority Data:</b> 08/184,140      21 January 1994 (21.01.94)      US <b>(71) Applicants:</b> THE UNITED STATES OF AMERICA, represented by THE SECRETARY OF THE NAVY [US/US]; William, Garvert, Chief of Naval Research (Code OOCB), Balston Tower 1, 800 N. Quincy Street, Arlington, VA 22217-5660 (US). CELLCO, INC. [US/US]; 12321 Middlebrook Road, Germantown, MD 20874 (US). <b>(72) Inventors:</b> DAVIS, Thomas, A.; 4137 Weeping Willow Court, Chantilly, VA 22021 (US). LEE, Kelvin, P.; 4628 Woodfield Road, Bethesda, MD 20814 (US). KIDWELL, William, R.; 10905 Lowell Court, Ijamsville, MD 21754 (US). <b>(74) Agents:</b> GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox, Suite 600, 1100 New York Avenue, Washington, DC 20005-3934 (US).		<b>(81) Designated States:</b> AU, CA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> HEMATOPOIETIC CELL EXPANSION AND TRANSPLANTATION METHODS  <b>(57) Abstract</b>  The application concerns a method for the <i>ex vivo</i> expansion of hematopoietic cells, including hematopoietic stem and progenitor cells, using endothelial support cells in an artificial capillary system (ACS). The invention also concerns improved bone marrow transplantation methods. Also, the application concerns a method for enhancing the <i>ex vivo</i> expansion of hematopoietic cells by coating the capillaries of an ACS with a suitable coating reagent. A method for transducing <i>ex vivo</i> expanding hematopoietic cells, including hematopoietic stem and progenitor cells, with packaged recombinant retrovirus vectors is also provided.		

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## Hematopoietic Cell Expansion and Transplantation Methods

This invention was made with government support. The government has certain rights in the invention.

### *Field of the Invention*

5           The invention relates to the *ex vivo* expansion of hematopoietic cells, including hematopoietic stem and progenitor cells. The invention further relates to improved bone marrow transplantation methods. The invention also relates to enhancing *ex vivo* expansion of hematopoietic cells by coating the capillaries of an artificial capillary system with a suitable coating reagent.  
10           Finally, the invention relates to methods for transducing the hematopoietic cells with retrovirus vectors.

### *Background of the Invention*

          Hematopoiesis, the production of mature blood cells, is a complex scheme of multilineage differentiation. Mature blood cells are derived from pluripotent hematopoietic stem cells (HSC). The defining characteristics of  
15           HSC are the capacity for extensive self-renewal and retention of multilineage differentiation potential (i.e., the ability to reconstitute the hematopoietic system). HSC proliferate and differentiate to produce progenitor cells, which in turn form precursor cells, which differentiate to form mature blood cells.

20           During ontogeny, hematopoiesis moves from yolk sac to liver/spleen and then to the bone marrow (Tavassoli, M., *Blood Cells* 17:269 (1991)). During early fetal life, hematopoiesis occurs within the liver and spleen. In the latter part of gestation, bone marrow spaces begin to develop and expand. HSC then migrate from liver/spleen to the bone marrow occupying "niches"  
25           in the developing marrow (Zanjani *et al.*, *J. Clin. Invest.* 89:1178 (1992)). Hematopoiesis subsequently primarily occurs in the bone marrow (Gordon *et al.*, *Bone Marrow Transplant* 4:335 (1989)).

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HSC are contained in a subpopulation of hematopoietic cells phenotypically characterized by the presence of the CD34 and absence of the CD38 cell surface antigens. Thus, undifferentiated pluripotent HSC capable of long term hematopoietic reconstitution are CD34<sup>+</sup>CD38<sup>-</sup>. *In vivo*, the bone marrow stroma (comprised of adipocytes, macrophages, fibroblasts, endothelial cells, and the associated extra-cellular matrix) provides the microenvironment for constitutive hematopoiesis (Gordon *et al.*, *Bone Marrow Transplant* 4:335 (1989)).

Bone marrow transplantation (BMT) has become an important therapeutic option for a number of conditions. For example, BMT is used in attempts to restore hematopoietic function following ablative chemotherapeutic conditioning regimens. Moreover, BMT has been used successfully to treat a number of congenital hematopoietic and metabolic disorders (Sullivan, K.M., *Transplant Proc.* 21 (Suppl. 1):41 (1989)).

However, conventional BMT has significant limitations. Currently, HSC used for clinical transplantation in the treatment of malignancies are usually contained in harvested adult bone marrow or peripheral blood. Most transplants done presently utilize a matched bone marrow donor (allogeneic BMT). Unfortunately, only about 10% of candidates for allogeneic BMT will actually have a human leukocyte antigen (HLA)-identical family member. This precludes an optimal donor for the majority of patients (Flake *et al.*, *Exp. Hemat.* 19:1061 (1991)). Moreover, results using HLA-mismatched donors have been disappointing (O'Reilly *et al.*, *Immunodef. Rev.* 1:23 (1990); Anasetti *et al.*, *N. Engl. J. Med.* 320:197 (1989); Ferrara *et al.*, *N. Engl. J. Med.* 324:667 (1991)).

In an attempt to overcome the necessity of finding a matched donor, autologous BMT has been developed in which the patients own marrow is harvested and then reinfused back into the patient after the marrow ablating event. However, harvesting the bone marrow still requires inpatient hospitalization and a surgical procedure. Moreover, the patients marrow is frequently damaged from prior anti-cancer treatment or contaminated with

malignant cells (Gale *et al.*, *Bone Marrow Transplant* 7:153 (1991)). This shortage of autologous marrow is even more problematic when multiple cycles of chemotherapy are required.

Thus, there has been much interest in the expansion of bone marrow cells *ex vivo* prior to transplantation (Edgington S.M., *Biotechnology* 10:1099 (1992)). Successful *ex vivo* expansion of HSC would allow transplantations in situations where, using currently available technology, adequate amounts of bone marrow cannot be harvested from the patient.

Two types of hematopoietic culture systems have been developed: stromal/hematopoietic cell co-culture systems (e.g., Dexter-type cultures), and liquid cultures which include hematopoietic cells and cytokines without stromal support cells. Dexter-type long term bone marrow cultures (LTBMCs) were developed in the 1970s. LTBMCs provided stable *ex vivo* hematopoietic systems for several months (Dexter *et al.*, *J. Cell Physiol.* 91:335 (1977); Dexter *et al.*, in: Wright, D.G., Greenberger, J.S. (Eds.): Long-term Bone Marrow Culture. New York, NY, Liss, p. 57 (1984)). However, LTBMCs exhibit exponentially decreasing numbers of total and progenitor cells with time, rendering the cultures unsuitable for cell expansion for clinical use (Eaves *et al.*, *J. Tissue Cult. Methods* 13:55 (1991)). In contrast, hematopoietic cells grown in liquid culture result in a large degree of nonadherent cell expansion (Haylock *et al.*, *Blood* 80:1405 (1992); Brandt *et al.*, *Blood* 79:634 (1992)). However, cellular differentiation and depletion of primitive cells (e.g., HSC) invariably occurs in these systems. For example, after culture of enriched primitive hematopoietic cells, measurements of long-term culture-initiating cells (LTC-IC) were reported as being below the input value (Sutherland *et al.*, *Blood* 78:666 (1991); Verfaillie, C.M., *Blood* 79:2821 (1992)).

Attempts to overcome these problems using a continuous perfusion culture of mononuclear cell (MNC) populations obtained from adult marrow without enrichment for CD34<sup>+</sup> cells resulted in expansion of progenitor cells (Koller *et al.*, *Blood* 82:378 (1993); Koller *et al.*, *Biotechnology* 11:358

(1993); Palsson *et al.*, *Biotechnology* 11:368 (1993)). However, these studies are limited because the cells were characterized only by the LTC-IC assay, and not by phenotype analysis or an *in vivo* model of engraftment. The LTC-IC, high proliferative potential-colony-forming cells (HPP-CFC), and blast colony-forming cells (CFU-B1) assays identify a primitive hematopoietic cell (Williams, D.A., *Blood* 81(12):3169 (1993)). However, the primitive cells identified by these assays include not only HSC but also more differentiated progenitor cells. Therefore, the extent of HSC identification may be overstated when these assays are used for characterization.

It is difficult to analyze factors and conditions affecting hematopoiesis in these co-culture systems due to the heterogeneity of both the hematopoietic cells (when MNC are used) and the supporting stromal cell layer. The successful use of a homogenous cell population to support the *ex vivo* expansion of HSC would greatly facilitate the study of hematopoiesis. However, to date, an *ex vivo* system utilizing homogenous support cells capable of achieving significant HSC expansion (without HSC depletion) has not appeared in the literature.

### *Summary of the Invention*

Bone marrow transplantation (BMT) therapies have been used for treating patients suffering from a variety of disorders. However, for allogeneic BMT, only about 10% of candidates for BMT have a human leukocyte antigen (HLA)-identical family member. This precludes an optimal donor for the majority of patients. Moreover, the difficulty in obtaining sufficient amounts of a patient's own marrow often precludes the use of autologous BMT.

These limitations have been overcome, at least in part, by the present invention, which provides a method for expanding hematopoietic cells, including hematopoietic stem and progenitor cells, *ex vivo* in an artificial capillary system (ACS) cartridge. By the invention, a substantially

homogenous population of endothelial cells is used to support hematopoietic cellular expansion. The method involves inoculating endothelial cells capable of supporting expansion of hematopoietic cells into an ACS cartridge, inoculating CD34<sup>+</sup> cells into the ACS cartridge, perfusing the ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of hematopoietic cells, and culturing the CD34<sup>+</sup> cells in the ACS cartridge for a sufficient amount of time to achieve hematopoietic cell expansion, including expansion of hematopoietic stem and progenitor cells. After culture, or at intervals during culture, nonadherent hematopoietic cells can be harvested from the ACS cartridge. Hematopoietic stem cells (HSC) and committed progenitor cell populations can be detected in the harvested nonadherent cells using flow cytometry and colony-forming assays.

The invention is also directed to improved bone marrow transplantation methods. The invention involves inoculating endothelial cells capable of supporting expansion of hematopoietic cells into an ACS cartridge, inoculating CD34<sup>+</sup> cells into the ACS cartridge, perfusing the ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of hematopoietic cells, culturing the CD34<sup>+</sup> cells in the ACS cartridge for a sufficient amount of time to achieve expansion of a therapeutically effective number of hematopoietic cells, including expansion of hematopoietic stem and progenitor cells, harvesting the cultured cells from the ACS cartridge, and transplanting the hematopoietic cells to a patient in need of a transplant.

The transplantation methods of the present invention are useful for autologous and allogeneic bone marrow transplants. The invention is particularly useful for autologous transplants to patients suffering from malignancies. Autologous marrow obtained from patients with malignancies are sometimes contaminated with tumor cells. Agents used to purge the autologous marrow are known to deplete hematopoietic stem and progenitor cells which results in delayed engraftment upon reinfusion. The present

invention overcomes this problem, at least in part, by replenishing and significantly expanding the hematopoietic stem and progenitor cell populations of purged marrow by *ex vivo* culture.

5 The invention further provides a method for enhancing the *ex vivo* expansion of hematopoietic cells by culturing the cells in an ACS cartridge having capillaries coated with a suitable coating reagent such as gelatin, collagen, fibronectin, or preferably, the adhesion protein described herein.

10 The invention also provides a method for transducing *ex vivo* expanding hematopoietic cells, including hematopoietic stem and progenitor cells, with a packaged recombinant retrovirus vector. The method involves inoculating an ACS cartridge with endothelial cells, CD34<sup>+</sup> cells, and a suspension containing packaged retroviral vectors, perfusing the cartridge with at least one hematopoietic growth factor, and culturing the CD34<sup>+</sup> cells in the presence of the packaged retrovirus vectors for a sufficient amount of time to achieve  
15 expansion and transduction of hematopoietic cells, including hematopoietic stem and progenitor cells. The retroviral vector can contain a heterologous gene encoding a therapeutically effective product. After expansion and harvesting, the transduced hematopoietic cells can be administered to a patient using the transplantation methods of the invention.

### 20 *Brief Description of the Drawings*

Figure 1: Comparison of Flask vs ACS culture of hematopoietic cells. Purified CD34<sup>+</sup> cells were co-cultured with PMVEC in either tissue culture flasks or the ECS of an ACS cartridge. After 7 days of expansion, nonadherent cells from the flask and ACS cultures were harvested and  
25 subjected to immunophenotyping with monoclonal antibodies against CD34 and CD38 cell surface antigens. The number of input CD34<sup>+</sup> cells, and the number of cells staining CD34<sup>+</sup>, CD34<sup>+</sup>CD38<sup>-</sup>, and CD34<sup>+</sup>CD38<sup>+</sup> after 7 days of either flask or ACS culture are shown graphically.

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Figure 2:  $1 \times 10^8$  PMVEC were inoculated into the ECS of an ACS cartridge. After one week,  $4 \times 10^6$  CD34<sup>+</sup> cells were also inoculated into the ECS. The culture was continuously maintained for 78 days. Total nonadherent cell production was enumerated weekly using a hemacytometer. Cumulative nonadherent cell yield generated in the ACS cartridge over 78 days is shown graphically.

Figure 3:  $1 \times 10^8$  PMVEC were inoculated into the ECS of an ACS cartridge. After one week,  $4 \times 10^6$  CD34<sup>+</sup> cells were also inoculated into the ECS. The culture was continuously maintained for 35 days. Total CD34<sup>+</sup> cell production was enumerated weekly by flow cytometry. Cumulative CD34<sup>+</sup> cell yield in the ACS cartridge over 35 days is shown graphically.

Figure 4:  $1 \times 10^8$  PMVEC were inoculated into the ECS of an ACS cartridge. After one week,  $4 \times 10^6$  CD34<sup>+</sup> cells were also inoculated into the ECS. The culture was continuously maintained for 35 days. Total CFU-GM cell production was enumerated weekly by a methylcellulose colony forming cell (CFC) assay. Cumulative CFU-GM cell yield in the ACS cartridge over 35 days is shown graphically.

Figure 5:  $3 \times 10^7$  PMVEC were inoculated into the lumen of ACS capillaries and allowed to adhere tightly to the luminal surface.  $1 \times 10^6$  CD34<sup>+</sup> cells were then inoculated into the ECS of the ACS cartridge. The culture was continuously maintained for 14 days. Total nonadherent cell production was enumerated weekly using a hemacytometer. Cumulative nonadherent cell yield in the ACS cartridge over 14 days is shown graphically. Total CFC number and lactate production over 14 days of culture are provided in Figures 6 and 7 respectively.

Figure 6: Aliquots of the CD34<sup>+</sup> cells taken prior to culture and of the nonadherent cells taken after 14 days of culture were assayed for CFC number. Total CFC number over 14 days of culture is shown graphically.

Figure 7: The lactate production by the cell culture determined by sampling the perfusate during the course of the culture using an automated analyzer is shown graphically.

Figure 8:  $33 \times 10^6$  mononuclear cells from bone marrow were inoculated into the ECS of cartridges containing capillaries either with or without a recombinant adhesion protein coating. Nonadherent cells were harvested after 12 days of culture. CFC assays were performed to determine the number of CFU-GM + BFU-E hematopoietic progenitor cell subtypes per 200,000 harvested nonadherent cells. A comparison between cell expansions achieved using coated and uncoated capillaries is shown graphically. Results from a glucose utilization assay are provided in Figure 9.

Figure 9: After 4 and 6 days of culture, aliquots from the ACS reservoir medium were taken and assayed for glucose consumption (grams/24 hr). The results are shown graphically.

Figure 10: The amino acid sequence (SEQ ID NO. 1) of a recombinant adhesion protein is provided. The adhesion protein was designed using two oligopeptide blocks, one providing the structural properties of silk fibroin protein and the other providing the cell attachment activity of human fibronectin.

### ***Detailed Description of the Invention***

The invention provides a method for the *ex vivo* expansion of hematopoietic cells, including hematopoietic stem and progenitor cells. The method involves inoculating endothelial cells capable of supporting expansion of hematopoietic cells into an artificial capillary system (ACS) cartridge, inoculating CD34<sup>+</sup> cells into the ACS cartridge, perfusing the ACS cartridge with culture medium containing at least one hematopoietic growth factor, and culturing the CD34<sup>+</sup> cells for a sufficient amount of time to achieve

hematopoietic cell expansion, including expansion of hematopoietic stem and progenitor cells.

Previously reported culture systems utilizing bone marrow cells enriched for CD34<sup>+</sup> cells reported cellular differentiation and depletion of the more primitive (stem and progenitor) cell populations (Sutherland *et al.*, *Blood* 78:666 (1991); Verfaillie, C.M., *Blood* 79:2821 (1992)). Thus, during expansion, the CD34<sup>+</sup> cells differentiated into more mature cells. Attempts by others to overcome this problem involved culturing bone marrow mononuclear cell (MNC) populations on monolayers of irradiated marrow stroma (a heterogeneous cell mixture including adipocytes, macrophages, fibroblasts, and endothelial cells) without prior enrichment for CD34<sup>+</sup> cells. This resulted in the expansion of progenitor cells. (Koller *et al.*, *Blood* 82:378 (1993); Koller *et al.*, *Biotechnology* 11:358 (1993); Palsson *et al.*, *Biotechnology* 11:368 (1993)). These studies suggest that a heterogeneous cell mixture (i.e., the endogenous marrow stroma) is necessary to support *ex vivo* expansion of hematopoietic stem and progenitor cells.

In contrast to previous studies, the present inventors have discovered that co-culturing CD34<sup>+</sup> cells and a substantially homogenous population of endothelial cells in an ACS cartridge achieves significant expansion of hematopoietic stem and progenitor cells. Moreover, in addition to hematopoietic stem and progenitor cell expansion, the present *ex vivo* culturing system supports the expansion and long-term maintenance of the entire hematopoietic system (stem, progenitor, precursor, and mature cells).

The invention encompasses co-culturing CD34<sup>+</sup> cells and endothelial cells in an ACS cartridge. The CD34<sup>+</sup> cells can be inoculated into the extra-capillary space (ECS) of an ACS cartridge as a subpopulation of cells included within a mixed cell population. Alternatively, the CD34<sup>+</sup> cells can be inoculated into the ECS of an ACS cartridge as an enriched population of CD34<sup>+</sup> cells.

Umbilical cord blood cells, peripheral blood cells, and bone marrow cells of mammals, including humans, nonhuman primates, and mice, are

mixed cell populations which include CD34<sup>+</sup> cells and can be obtained according to conventional techniques (Kennedy *et al.*, *J Natl Cancer Inst* 83 (13):921 (1991); Koller *et al.*, *Biotechnology* 11:358 (1993)). Mononuclear cells (MNC) (which include CD34<sup>+</sup> cells) can be prepared from umbilical cord blood cells, peripheral blood cells, and bone marrow cells using Ficoll-Hypaque density gradient centrifugation as described in Example 3. Preparing MNC from cord blood and bone marrow using Ficoll-Hypaque density gradient centrifugation is also described in Koller *et al.*, *Blood* 82:378 (1993) and Koller *et al.*, *Biotechnology* 11:358 (1993), respectively. Approximately 10<sup>5</sup>-10<sup>7</sup> of the above MNC can be inoculated into the ECS of an ACS cartridge for *ex vivo* expansion of hematopoietic cells.

An enriched population of CD34<sup>+</sup> cells can be prepared from umbilical cord blood cells, peripheral blood cells, and bone marrow cells from the above-described mammals according to conventional techniques. For example, the anti-CD34<sup>+</sup> monoclonal antibody cell sorting techniques described in Brandt *et al.*, *J Clin Invest* 86:932 (1990); Edgington, S.M., *Biotechnology* 10:1099 (1992); and Srour *et al.*, *Blood* 81(3):661 (1993) can be used to obtain an enriched population of CD34<sup>+</sup> cells. Similarly, the avidin-biotin immunoaffinity process described in Berenson *et al.*, *J Immunol Meth* 91: 11 (1986); Berenson *et al.*, *Blood* 67 (2):509 (1986); and Berenson *et al.*, *Blood* 69 (5):1363 (1987) can also be used for enrichment of CD34<sup>+</sup> cells. In a preferred embodiment, CD34<sup>+</sup> cells are enriched from bone marrow cells according to the positive immunomagnetic selection technique described in Example 1.

The CD34<sup>+</sup> cells, as either an enriched or mixed cell population, are inoculated into the ECS of an ACS cartridge or cryopreserved and stored under liquid nitrogen for future use. Prior to inoculation into an ACS cartridge, it is preferable to resuspend the CD34<sup>+</sup> cells in a suitable culture medium. However, if frozen, cryopreserved CD34<sup>+</sup> cells should first be thawed using standard techniques. For example, the cells can be thawed

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rapidly at 37°C and diluted in a suitable prewarmed (37°C) culture medium. Suitable culture mediums are described below.

The cells are resuspended in a suitable culture medium at an appropriate concentration. For example, a concentration of  $1 \times 10^6$  CD34<sup>+</sup> cells/ml can be used. Other suitable concentrations can be determined empirically. The CD34<sup>+</sup> cells are then ready for inoculation into the ECS of an ACS cartridge. If an enriched population of CD34<sup>+</sup> cells is used, as few as  $10^2$  -  $10^3$  CD34<sup>+</sup> cells can be inoculated into the ECS for *ex vivo* expansion. Preferably, however,  $10^5$ - $10^7$  CD34<sup>+</sup> cells are inoculated.

As indicated, by the invention, CD34<sup>+</sup> cells are co-cultured with endothelial cells in an ACS cartridge. Endothelial cells which, in conjunction with at least one hematopoietic growth factor (described below), are capable of supporting expansion of hematopoietic stem and progenitor cells are suitable for use in the invention. For example, endothelial cells derived from the central nervous system (including the brain) of mammals such as humans, nonhuman primates, pigs and mice can be used to support hematopoietic cell expansion. Furthermore, endothelial cells derived from other mammalian organs (such as bone marrow or fetal cells) may also support hematopoietic cell expansion. Preferably, the endothelial cells are derived from the central nervous system of pigs. More preferably, the endothelial cells are porcine brain microvascular endothelial cells (PMVEC). Thus, by the invention, the cells used to support expansion of the hematopoietic cells constitute a substantially homogenous cell population.

Suitable endothelial cells can be isolated from mammals according to conventional techniques. For example, PMVEC can be isolated from the brains of pigs using the procedure described in Example 1. After isolation, the cells can be grown to confluency on culture plates to provide an accessible source of substantially homogenous endothelial support cells.

As discussed above, in the present invention, the endothelial cells, in conjunction with at least one hematopoietic growth factor, support expansion of hematopoietic cells, including hematopoietic stem and progenitor cells.

Thus, a sufficient number of endothelial cells must be inoculated into the ACS cartridge to support expansion. The number of endothelial cells that are "sufficient" to support expansion can be determined empirically. The inventors have discovered that inoculating approximately  $10^6$ - $10^8$  endothelial cells into the ACS cartridge is sufficient to support expansion of the hematopoietic cells. Preferably,  $10^7$ - $10^8$  endothelial cells are inoculated. The endothelial cells can be inoculated into the ACS cartridge before, after, or simultaneously with the CD34<sup>+</sup> cells. Preferably, however, the endothelial cells are inoculated prior to CD34<sup>+</sup> cell inoculation. To support hematopoietic cell expansion, the endothelial cells are immobilized on the outer capillary wall. In an alternative embodiment, the endothelial cells are immobilized on the inner capillary wall.

Immobilization of endothelial cells on the outer capillary wall can be achieved simply by injecting the endothelial cells into the ECS and dispersing them over the hollow capillaries by flushing culture medium back and forth through the two sampling side-ports using syringes. After culturing the cells for a sufficient amount of time (approximately 1-3 days), nonadherent endothelial cells which have not settled onto the outer capillary wall can be removed by flushing the ECS with culture medium. This process leaves endothelial cells which are immobilized on the outer capillary wall and purges nonadherent endothelial cells from the ECS.

Immobilization of endothelial cells onto the inner capillary wall can be achieved by injecting the endothelial cells into the capillary lumen via the ACS endport. This is followed by culturing the endothelial cells in the capillary lumen for a sufficient amount of time (approximately 1-3 days) to allow the cells to adhere onto the inner capillary wall. The cartridge can then be perfused with culture medium to remove nonadherent cells leaving only endothelial cells which are immobilized to the inner capillary wall.

Hematopoietic growth factors capable of stimulating expansion of hematopoietic cells, including hematopoietic stem and progenitor cells, are described in the literature (Moore *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7134

(1987); Leary *et al.*, *Blood* 71:1759 (1988); Brandt *et al.*, *J. Clin. Invest.* 86:932 (1990); Kobayashi *et al.*, *Blood* 78:1947 (1991); Meunch *et al.*, *Blood* 81:3463 (1993); Bernstein *et al.*, *Blood* 77:2316 (1991); and Bodine *et al.*, *Blood* 79:913 (1992)). In particular, the following growth factors can be used to stimulate expansion of hematopoietic cells: interleukin-1 (IL-1), interleukin-1 $\alpha$  (IL-1 $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-11 (IL-11), erythropoietin, leukemic inhibitory factor (LIF), PIXY-321, and stem cell factor (SCF). These hematopoietic growth factors are readily available and can be used alone or in combinations of two or more. Preferably, the growth factors used for stimulating expansion of the hematopoietic cells are GM-CSF, IL-3, SCF, and IL-6.

Any culture medium recognized in the art as appropriate can be used to perfuse the ACS cartridge. In addition to one or more hematopoietic growth factors, the culture medium can be supplemented with serums such as fetal calf serum (FCS) and antibiotics such as Penicillin, Streptomycin, and Amphotericin B. A particularly suitable medium is Isocove's Modified Dulbecco's Medium (IMDM). IMDM can be supplemented with 10% fetal bovine serum (FBS), 100 ug/mL L-glutamine, and 100 U/mL penicillin/streptomycin. This is referred to herein as complete culture medium. IMDM is readily available as are other tissue culture media.

As discussed above, the culture medium will also contain at least one hematopoietic growth factor. Hematopoietic growth factors should be included in the culture medium at concentrations of approximately 0.1-500 ng/ml, preferably at concentrations of approximately 1-150 ng/ml. However, as the skilled artisan will recognize, other concentrations can be used as required.

After culture, or at periodic intervals during culture, nonadherent hematopoietic cells can be harvested from the ACS cartridge simply by flushing the cartridge's extra-capillary space (ECS) with fresh medium or by gently shaking the cells from the cartridge. A small number of cells remain

behind which re-seed the ACS for further culture. By "extra-capillary space" (ECS) is intended the space wherein the cells grow within the shell of the ACS that is external to the semi-permeable capillaries. After harvesting, the suspension containing expanded cells can be pelleted by centrifugation (400-600 g) and the cells resuspended at desired concentrations in culture medium for further use.

After culture, a small number of primitive hematopoietic cells (e.g., HSC and progenitor cells) may adhere to the endothelial support cells. These hematopoietic cells can be recovered by trypsinization and separated from other adherent cells using positive selection with anti-CD34<sup>+</sup> antibodies as described above.

The inoculated CD34<sup>+</sup> cells should be cultured for a sufficient amount of time to achieve hematopoietic cell expansion, including expansion of hematopoietic stem and progenitor cells. The amount of time that is "sufficient" to achieve expansion can easily be determined empirically. For example, the phenotype analysis techniques described in Example 1 are useful for determining the amount of culturing time required to achieve cellular expansion. The inventors have discovered that culturing for 5 days achieves significant primitive hematopoietic cell expansion. Moreover, the inventors achieved a 15-fold expansion of CD34<sup>+</sup> cells and a 70-fold expansion of CD34<sup>+</sup>CD38<sup>-</sup> HSC after only 7 days of culture. If nonadherent cells are harvested periodically (for example, weekly), then culturing can occur for an extended period of time. For example, the inventors have achieved long-term and steady hematopoiesis during 78 days of culture. Thus, it will be recognized that different culture durations can be used depending on the exigencies of each experiment.

An ACS cartridge consists of an outer shell casing that is biocompatible with the growth of mammalian cells, a plurality of semi-permeable hollow capillaries encased within the shell that are also biocompatible with the growth of mammalian cells on or near them, and the ECS, which contains the cells and the ECS cell supernatant.

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Tissue culture medium flows within the capillary lumens and is also included within the shell surrounding the capillaries. The tissue culture medium, which may differ in these two compartments, contains diffusible components that are capable of expanding hematopoietic cells. The medium is provided in a reservoir from which it is pumped through the capillaries. The flow rate can be controlled by varying the rpm of the pump head.

ACS are described in Knazek *et al.*, U.S. Patent Nos. 4,220,725, 4,206,015, 4,200,689, 3,883,393, and 3,821,087. A typical ACS consists of a standard glass media bottle, which serves as the reservoir, a pump, a hollow fiber bioreactor, which consists of the capillaries and shell casing in which cells are cultured, and medical grade silicone rubber tubing, or other connecting means, which serves as a gas exchanger to maintain the appropriate pH and PO<sub>2</sub> of the culture medium. The reservoir can also be a plastic bag. All components are secured to a tray of sufficiently small dimensions to fit within a standard tissue culture incubator chamber. The pump speed is determined by an electronic control unit which is placed outside of the incubator and is connected to the pump motor via a cable which passes through the gasket of the incubator door. The pump motor can be magnetically coupled to the pump, being lifted from the system prior to steam autoclaving. The pump motor can also drive a cam which moves a pin means which, in turn, compresses tubing and causes unidirectional flow through a series of one-way valves.

Tissue culture medium is drawn from the reservoir and pumped through the gas exchange tubing in which it is reoxygenated and its pH readjusted and then through the lumen of the hollow capillaries prior to returning to the reservoir for subsequent recirculation. The order of sequences may be altered without substantially changing the functionality.

The entire system is sterilized prior to operation and is designed for operation in a standard air-CO<sub>2</sub> tissue culture incubator. The flow rate can be increased as the number of cells increases with time. Typically the initial flow rate of the medium is adjusted to about 4 ml/min. Upon inoculation through

the ACS side ports, the cells settle onto the surface of the hollow capillaries, through the walls of which nutrients pass to feed the cells and through which metabolic waste products pass and are diluted into the large volume of the recirculating perfusate. The selected capillaries should be semi-permeable or microporous to permit the passage of nutrients into the ECS by diffusion or bulk transfer and should be of a material on which or in the vicinity of which the cells are able to grow. The capillaries are made of material, such as cellulose diacetate or polypropylene or other suitable material, that is semi-permeable or porous and suitable for the growth of mammalian cells. It may be necessary to treat the surface of certain types of capillaries with reagents to enable some cells to adhere to the surface. For example, polypropylene capillaries 15 cm in length, having 0.5  $\mu$  pores are suitable for use in practicing the invention.

As discussed above, after culture, or at periodic intervals during culture, nonadherent cells can be harvested from the ACS cartridge for enumeration and analysis. The harvested nonadherent cells can be enumerated and immunophenotyped using a hemacytometer and immunofluorescence staining according to conventional techniques. For example, flow cytometric analysis of subpopulations of cells labeled with monoclonal antibodies specific for cluster determinants (CD) antigens can be used. These CD antigens can be labeled using commercially available monoclonal antibodies conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Monoclonal antibodies specific for the CD34, CD38, CD20, CD14, CD3, CD4, CD16, CD15, HLA-DR, CD33, CD11b and CD8 antigens can be obtained from Becton Dickinson Monoclonals, San Jose, CA. After labelling the harvested nonadherent cells with the above monoclonal antibodies, flow cytometric analysis can then be performed to determine whether subpopulations of cells bearing the CD antigens are present in the sample. Thus, CD34<sup>+</sup> cells, CD34<sup>+</sup>CD38<sup>+</sup> cells and CD34<sup>+</sup>CD38<sup>-</sup> HSC are determined in the harvested nonadherent cells by labelling the cells with anti-CD34 and anti-CD38 monoclonal antibodies and performing flow cytometry.

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The presence of hematopoietic progenitor subpopulations (multipotent colony forming units [CFU-MIX]; colony forming unit-granulocyte/macrophage [CFU-GM]; erythroid burst-forming units [BFU-E]; blast-colony forming units [CFU-Blast]; and megakaryocyte colonies [CFU-Mk]) in the harvested nonadherent cells can be determined by conventional techniques (Meisenberg *et al.*, *Blood* 79:2267 (1992)). For example, methylcellulose CFC (colony-forming cell) assays can be used.

The *ex vivo* culture system of the present invention preferentially supports expansion (from inoculated CD34<sup>+</sup> cells) of primitive hematopoietic stem cells (HSC) having the phenotypic markers CD34<sup>+</sup>CD38<sup>-</sup>. CD34<sup>+</sup>CD38<sup>-</sup> HSC include primitive pluripotent cells capable of self-renewal, multilineage differentiation and reconstitution of the hematopoietic system. Thus, contrary to previously reported attempts where *ex vivo* culture of CD34<sup>+</sup> cells resulted in depletion of stem cells, the present invention provides an *ex vivo* method for expanding CD34<sup>+</sup>CD38<sup>-</sup> HSC to substantial numbers.

For example, the results provided in Example 1 (displayed graphically in Figure 1) show that after only 7 days of culture in the *ex vivo* system of the present invention, there is a 15-fold expansion of CD34<sup>+</sup> cells. The purity of the starting CD34<sup>+</sup> cell population was 85% CD34<sup>+</sup>CD38<sup>+</sup> and 15% CD34<sup>+</sup>CD38<sup>-</sup>. After 7 days of culture, the absolute number of CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> cells increased 6.2-fold and 70.5-fold, respectively. Thus, by the invention, substantial expansion of undifferentiated and uncommitted hematopoietic stem cells (CD34<sup>+</sup>CD38<sup>-</sup> HSC) occurs after only 7 days of culture. The inventors also co-cultured the CD34<sup>+</sup> cells and the endothelial cells in flask culture. However, in flask culture, only a 11-fold expansion of CD34<sup>+</sup> cells occurred. Moreover, the absolute number of CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> cells increased 6.7-fold and 35.7-fold, respectively.

Thus, these data demonstrate that less differentiation and depletion of primitive CD34<sup>+</sup>CD38<sup>-</sup> HSC occur during *ex vivo* culture using the ACS/endothelial cell system of the present invention as compared to the flask/endothelial cell system.

The *ex vivo* expansion system of the present invention has a variety of uses. These include providing a rich source of transplantable hematopoietic stem and progenitor cells, facilitating retroviral transduction of hematopoietic stem and progenitor cells, *ex vivo* maintenance of transplantable cells during which time cells can be assayed for pathogenic contamination, and facilitating the study of factors and conditions affecting hematopoiesis.

Bone marrow transplantation (BMT) has become standard therapy for a number of conditions. These include intrinsic marrow defects such as congenital hematopoietic and metabolic disorders and bone marrow injury due to ablative and nonablative conditioning regimens consequent to hematologic and non-hematologic malignancies (Sullivan, K.M., *Transplant Proc.* 21 (Suppl. 1):41 (1989)).

The invention improves on these applications of BMT therapy by providing a readily accessible source of *ex vivo* expanded hematopoietic cells, including hematopoietic stem and progenitor cells. Thus, the invention is further directed to a method for transplanting *ex vivo* expanded hematopoietic cells, including hematopoietic stem and progenitor cells, to a patient. The method involves inoculating endothelial cells capable of supporting expansion of hematopoietic cells into an ACS cartridge, inoculating CD34<sup>+</sup> cells into the ACS cartridge, perfusing the ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of hematopoietic cells, culturing the CD34<sup>+</sup> cells for a sufficient amount of time to achieve expansion of a therapeutically effective number of hematopoietic cells, including hematopoietic stem and progenitor cells, harvesting cultured cells from the ACS cartridge, and transplanting the hematopoietic cells to a patient.

Methods for expanding, harvesting, and, optionally storing (by cryopreservation) hematopoietic cells using an ACS are discussed above. After harvesting (or thawing after storage), the hematopoietic cells, including hematopoietic stem and progenitor cells, can be transplanted to a patient according to conventional techniques (Kennedy *et al.*, *J Natl Cancer Inst*

83(13):920 (1991); Touraine *et al.*, *Thymus* 10:75 (1987)). For example, the cells can be transplanted by intravenous infusion.

Short-term reconstitution of the hematopoietic system is necessary to successfully treat patients suffering from cytopenia following non-ablative chemotherapy. Approximately  $2 \times 10^5$  CFU-GM progenitor cells per Kg patient body weight are required for short term reconstitution of the hematopoietic system. Thus, for a 70 Kg adult patient, approximately  $1.4 \times 10^7$  CFU-GM are needed. By the invention, approximately  $10^7$  CFU-GM can be generated *ex vivo* in only two weeks of culture from an inoculum of about  $10^6$  CD34<sup>+</sup> cells. This number of CD34<sup>+</sup> cells can be obtained from one 15 ml bone marrow aspirate taken on an outpatient basis. Thus, the invention provides a method for treating patients in need of short-term reconstitution of the hematopoietic system. As indicated, the amount of culture time that is "sufficient" to achieved a therapeutically effective number of cells is approximately two weeks. Of course, longer culture periods to achieve even greater expansions of committed progenitor cells and post-progenitor cells can also be used if needed.

Long-term reconstitution of a patient's hematopoietic system is required, for example, following high dose myeloablative chemotherapy. Since hematopoietic progenitor cells are "committed", transplantation of progenitor cells alone will not achieve long-term reconstitution of the hematopoietic system. Instead, patients must be infused with pluripotent HSC (CD34<sup>+</sup>CD38<sup>-</sup> cells) in order to achieve long-term reconstitution. Preferably, the pluripotent CD34<sup>+</sup>CD38<sup>-</sup> HSC will be co-transplanted with CFU-GM cells in order both to avoid early aplasia and to provide long-term engraftment. By the invention, a therapeutically effective number of CD34<sup>+</sup>CD38<sup>-</sup> HSC can be generated in only 7 days of culture. Preferably, the cells are cultured for at least 10-14 days to ensure that a therapeutically effective number of CD34<sup>+</sup>CD38<sup>-</sup> HSC have been generated. Different culture durations can be used as needed.

In the *ex vivo* culturing system of the present invention, subpopulations of both CD34<sup>+</sup>CD38<sup>-</sup> HSC and CFU-GM progenitors are present in nonadherent cells harvested from the ACS cartridge. In fact, after only two weeks of culture, a therapeutically effective number of both CD34<sup>+</sup>CD38<sup>-</sup> HSC and CFU-GM progenitors are present in the nonadherent cells. Thus, by the invention, nonadherent cells harvested from an ACS cartridge can be transplanted into a patient in need of either short-term or long-term hematopoietic reconstitution.

The present invention is useful for the *ex vivo* expansion of hematopoietic cells for use in autologous and allogeneic bone marrow transplants. In the latter, of course, graft vs host disease (GVHD) must be avoided and transplantation of HLA-compatible bone marrow is preferred. However, only about 10% of candidates for BMT have a human leukocyte antigen (HLA)-identical family member. This precludes an optimal donor for the majority of patients (Flake *et al.*, *Exp. Hemat.* 19:1061 (1991)).

Autologous marrow is frequently either damaged from prior anti-cancer treatment or contaminated with malignant cells (Gale *et al.*, *Bone Marrow Transplant* 7:153 (1991)). This shortage of autologous marrow is even more problematic when multiple cycles of chemotherapy are required.

Thus, the present invention meets a particularly pressing need in the art by providing a method for achieving therapeutically effective numbers of autologous hematopoietic stem and progenitor cells that are useful in BMT. For example, CD34<sup>+</sup> cells for use in the present invention can be obtained from bone marrow cells harvested by aspiration from patients suffering from a number of malignancies including leukemias, lymphomas, Hodgkin's disease, myeloma, myelofibrosis, and malignant breast cancers.

However, bone marrow cells harvested from patients suffering from a malignancy sometimes are contaminated with tumor cells (Kennedy *et al.*, *J Natl Cancer Inst* 83(13): 920 (1991); Thomas E.D., *J Clin Oncology* 1(9):517 (1983)). Considerable concern exists about possible reinfusion of viable cancer cells. Thus, after harvesting, if the bone marrow cells are

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contaminated, it is preferable that the cells are subjected to purging with a suitable tumor purging agent. One such agent is 4-hydroperoxycyclophosphamide (4-HC). However, incubation with 4-HC is known to reduce the frequency of pluripotent HSC and committed progenitor cells *in vitro* (Gordon *et al.*, *Leuk Res* 9:1017 (1985)). This has been reported to cause a delay in the engraftment of reinfused autologous marrow (Kaizer *et al.*, *Blood* 65:1504 (1985)). Bone marrow cells harvested from a patient suffering from a malignancy may also be purged by positive selection for CD34<sup>+</sup> cells using anti-CD34<sup>+</sup> antibody immobilized to a column matrix or magnetic beads.

As discussed above, culturing CD34<sup>+</sup> cells by the method of the present invention results in significant expansion of pluripotent hematopoietic stem and committed progenitor cells. Therefore, the delayed engraftment which occurs when purged autologous bone marrow is reinfused into a patient can be overcome by first culturing the purged cells according to the present invention prior to reinfusion.

Accordingly, the present invention also provides a method for overcoming the delay in engraftment caused by tumor purging agents which deplete pluripotent HSC and committed progenitor cells in autologous marrow. The method involves obtaining bone marrow from a patient suffering from a malignant tumor, purging the harvested marrow with a suitable tumor purging agent, and/or purifying CD34<sup>+</sup> cells, inoculating endothelial cells capable of supporting expansion of hematopoietic cells into an ACS cartridge, inoculating the CD34<sup>+</sup> cells into the ACS cartridge, perfusing the ACS cartridge with culture medium containing at least one hematopoietic growth factor, and culturing the CD34<sup>+</sup> cells for a sufficient amount of time to achieve expansion of a therapeutically effective number of hematopoietic cells, harvesting cultured cells from the ACS cartridge, and transplanting the cells into the patient.

As discussed above, the order of inoculating the CD34<sup>+</sup> and endothelial cells can be reversed or performed simultaneously. Moreover, the purging

step with a suitable tumor purging agent can also occur after harvesting expanded hematopoietic cells from the ACS cartridge. This will depend on experimental design.

5 The invention further provides a method for enhancing the *ex vivo* expansion of hematopoietic cells by coating the ACS capillaries with a suitable coating reagent. Suitable coating reagents include gelatin, collagen, fibronectin and, preferably, a polypeptide having an amino acid sequence substantially identical to that shown in Figure 10 (SEQ ID NO. 1). A polypeptide having an amino acid sequence substantially identical to that  
10 shown in Figure 10 is herein referred to as "the adhesion protein."

Coating reagents such as gelatin, collagen, and fibronectin are readily available to the skilled artisan and can be applied to the ACS capillaries using conventional techniques. The adhesion protein, which is the preferred coating reagent for use in the present invention, was designed using two oligopeptide  
15 blocks and is a highly active and stable substrate for receptor-specific cell attachment. The first oligopeptide block (16 amino acids in length) provides the cell attachment activity of human fibronectin (Cappello *et al. Polymer Reprints* 31:193 (1990)) and has the amino acid sequence Gly Ala Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly (SEQ ID NO. 2). The second  
20 oligopeptide block (6 amino acids in length) provides the structural properties of the silk fibroin protein and has the amino acid sequence Gly Ala Gly Ala Gly Ser (SEQ ID NO. 3). These two blocks are configured in a string where one "biological" block occurs after every nine "structural" blocks. This string is repeated 13 times to yield a polypeptide having 980 amino acids with a  
25 predicted molecular weight of 72,738 daltons (Figure 10). Thus, the adhesion protein incorporates thirteen Arg Gly Asp ligands modeled after the Arg Gly Asp sequence from human fibronectin interspersed between crystalline regions derived from natural silk. The adhesion protein can be produced recombinantly as described in Cappello J., *Materials Research Society Bulletin*  
30 17(10):48 (1992). Moreover, the adhesion protein is sold commercially under the trademark PRONECTIN™ F (Protein Polymer Technologies, Inc. San

Diego CA 92121)). Accordingly, the adhesion protein is readily available to the skilled artisan.

The adhesion protein is applied to the ACS capillaries simply by filling the ECS and capillary lumen of an ACS cartridge with a diluted adhesion protein solution. For example, a 1%-25% dilution of "stock" adhesion protein solution in phosphate buffered saline (PBS) is suitable for coating the ACS capillaries. (A "stock" solution of adhesion protein contains a concentration of approximately 1 mg/ml adhesion protein in a 4.5 molar lithium perchlorate solution ( $\text{LiClO}_4$ ).) After one hour, the ACS cartridge should be thoroughly washed with deionized water to remove adhesion protein not adhered to the capillary surface. The adhesion protein-coated capillaries are then ready for use in the *ex vivo* culturing system of the present invention. In addition to expanding hematopoietic cells from a  $\text{CD34}^+$  and endothelial cell co-inoculum as described above, an ACS cartridge having adhesion protein-coated capillaries is also useful for the *ex vivo* expansion of hematopoietic cells from an inoculum of mononuclear cells obtained from bone marrow, peripheral blood, or umbilical cord blood without enrichment for  $\text{CD34}^+$  cells.

Mononuclear cells (MNC) from bone marrow, peripheral blood, or umbilical cord blood can be prepared using Ficoll-Hypaque density gradient centrifugation as described above. The MNC are inoculated into the ECS of an ACS cartridge and cultured using the reagents and conditions described above. Example 3 provides a direct comparison of culturing MNC using capillaries with and without the adhesion protein coating. The results demonstrate that adhesion protein-coated capillaries provide an approximately 2.8-fold increase in hematopoietic progenitor cell growth (CFU-GM + BFU-E) and an approximately 1.8-fold increase in stromal cell activity as compared to capillaries without the adhesion protein coating. Thus, the invention further provides a method for enhancing the *ex vivo* expansion of hematopoietic cells by coating ACS capillaries with a suitable coating reagent such as gelatin, collagen, fibronectin, and preferably, the adhesion protein described herein.

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Active target cell cycling is required for retroviral integration (Nolta *et al.*, *Exp. Hematol.* 20:1065 (1992)). Thus, the invention further provides a method for transducing *ex vivo* expanded hematopoietic cells, including hematopoietic stem and progenitor cells, with retrovirus vectors. The method involves inoculating an ACS cartridge with endothelial cells capable of supporting expansion of hematopoietic cells, inoculating the ACS cartridge with CD34<sup>+</sup> cells, perfusing the ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of the hematopoietic cells, culturing the CD34<sup>+</sup> cells in the presence of the retrovirus vectors in the ACS cartridge for a sufficient amount of time to achieve hematopoietic cell expansion, and harvesting the cultured cells which have been transduced with the retrovirus vector.

Retrovirus vectors are the preferred vectors for genetic therapy (Anderson *et al.*, *Science* 226:401(1984)). This is because retrovirus infection is highly efficient and retrovirus vectors modified to be replication incompetent stably integrate into the host cell's genome. Packaging cell lines are capable of "packaging" the replication incompetent retrovirus vectors thereby rendering them infectious and therefore capable of transducing target cells. Such retrovirus vectors can stably integrate into the host cell genome upon transduction into a target cell.

U.S. Patent No. 4,861,719 (Miller, D.) describes the construction of various packaging cell lines including PA317 (ATCC Accession No. CRL 9078). PA317 is capable of packaging high concentrations of recombinant retrovirus vectors. Hock *et al.*, *Blood* 74:876 (1989) packaged high concentrations of LASN (a retrovirus vector containing the ADA and Neo resistance genes) in a cell line derived from PA317. Moreover, Knazek *et al.*, (abstract from presentation at BioEast 91 in Washington, DC, January 1991) showed that even more concentrated suspensions of packaged LASN are produced if the LASN-producing PA317 is grown to near solid tissue density with the CELLMAX<sup>TM</sup> 100 (Cellco, Inc.) artificial capillary system. This is important since highly concentrated suspensions of retroviral vectors are

necessary to efficiently transduce target cells. Thus, from the above, it is clear that several packaging systems are available that can be used to package recombinant retrovirus vectors to yield highly concentrated suspensions.

The retrovirus vector can be modified by inserting heterologous genes encoding therapeutically effective products. For example, LASN contains the ADA gene whose product is useful for treating a type of severe combined immunodeficiency disease (SCID). Other retrovirus vectors which can be modified by insertion of a heterologous gene encoding a therapeutically effective product are pN2 (Keller *et al.*, *Nature* 318:149(1985)); pLHL (Miller *et al.*, *Cold Spring Harbor Symp. on Quant. Bio.*, Vol. LI, Cold Spring Harbor Laboratory, p. 1013 (1986)); pSDHT (Miller *et al.*, *Somat. Cell. Mol. Genet.* 12:175 (1986)); pLPL (*Proc. Natl. Acad. Sci. USA* 80:4709). These vectors are known and available to the skilled artisan.

In particular, genes, which encode the following therapeutically effective products, can be inserted as heterologous genes into the recombinant retrovirus vectors using conventional techniques: ADA, Factor VIII, and Factor IX. These genes are known and available to the skilled artisan.

When recombinant retrovirus vector-producing packaging cells lines are grown in culture as described above, high concentrations of packaged recombinant retrovirus particles are produced in the cell supernatant. CD34<sup>+</sup> cells can be added to the vector-containing supernatant. The suspension, containing packaged recombinant-retrovirus vector and target CD34<sup>+</sup>, can then be inoculated into the ECS for expansion. The CD34<sup>+</sup> cells are then cultured in the ACS cartridge, in the presence of the packaged recombinant retrovirus vectors and endothelial cells, for a sufficient amount of time to achieve expansion of hematopoietic cells. Appropriate culturing time and conditions are described above. After culture, nonadherent transduced hematopoietic cells, including transduced hematopoietic stem and progenitor cells, are harvested from the ACS cartridge by flushing the cartridge with culture medium or by gently shaking the cells from the cartridge. After culture, a small number of transduced hematopoietic cells may also be bound to the

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endothelial support cells on the capillary surface. These hematopoietic cells can be recovered by trypsinization and separated from other adherent cells by positive selection with anti-CD34<sup>+</sup> antibodies as described above.

Alternatively, the endothelial cells and CD34<sup>+</sup> cells can be inoculated into the ACS cartridge (as described above) prior to addition of the packaged retrovirus vectors which are then added either before or after culture has been established. Also, the retrovirus vector-containing supernatant can be added to the ACS cartridge prior to inoculation of endothelial cells and CD34<sup>+</sup> cells. The order will depend on experimental design.

For efficient viral insertion into primitive hematopoietic cells, the cells must be actively dividing with limited differentiation. An inoculum of approximately  $4 \times 10^6$  CD34<sup>+</sup> cells includes approximately  $6 \times 10^5$  CD34<sup>+</sup>CD38<sup>-</sup> HSC. The inventors have discovered that, after only 7 days of culture using the ACS system of the present invention, an approximately 70-fold expansion of these CD34<sup>+</sup>CD38<sup>-</sup> HSC occurs. Thus, the present invention provides the conditions necessary for efficient viral insertion into primitive hematopoietic cells.

To increase the concentration of hematopoietic cells that are transduced with retrovirus vector, supernatant containing packaged retrovirus vector can be added at intervals during culture. For example, the vector-containing supernatant can be added to the cartridge every two days. The culture can be terminated after eight days. Of course, different intervals and volumes of supernatant can be used as needed.

The presence of the recombinant retrovirus vector in the expanded hematopoietic cells can be confirmed using the polymerase chain reaction (PCR). For example, for detection of a particular retrovirus vector, DNA primers flanking sequences specific to the vector (or heterologous gene insert) and not contained in the host cell genome can be used to amplify the retrovirus vector sequence (or heterologous gene sequence). The amplification product can then be loaded and run on a gel and probed with a labeled complementary DNA probe.

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The transduced hematopoietic cells, including transduced hematopoietic stem and progenitor cells, can then be transplanted into a patient using the transplantation methods described above.

Having generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration and are not intended to be limiting.

## ***Experimental***

### ***Example 1***

#### ***Ex Vivo Expansion of Hematopoietic Cells in an ACS***

##### ***Materials and Methods***

###### ***CD34<sup>+</sup> Bone Marrow Cells***

Human vertebral body bone marrow was procured from cadavers as described in the Naval Medical Research Institute publication no. 90-62 (available from the Defense Technical Information Center, AD# A226 538).

Briefly, marrow was obtained from the bone matrix by sterile techniques and placed in sterile culture support media. Low density mononuclear cells were separated over ficoll-Hypaque (specific gravity 1.077g/mL; Pharmacia Fine Chemicals, Piscataway, NJ) density gradients at 400 g for 30 min at 22°C. Low density cells at the interfaces were harvested, washed twice by centrifugation (400 g for 10 min) and resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) 100 ug/mL L-glutamine (Gibco, Grand Island, NY) and 100 U/mL penicillin/streptomycin (Gibco, Grand Island, NY). This culture medium is herein referred to as complete culture medium.

CD34<sup>+</sup> bone marrow progenitor cells were further purified by positive immunomagnetic selection using a monoclonal antibody specific for the CD34 antigen (K6.1).

5 The monoclonal antibody K6.1 was produced by fusing SP-2/0-AG14 plasmacytoma cells (American Type Culture Collection (ATCC), Rockville, MD) with splenocytes from a BALB/cByJ mouse (Jackson Laboratory, Bar Harbor, ME) which had been hyperimmunized with viable KG-1a cells (ATCC, Rockville, MD). Injections containing 10 to 20 million KG-1a cells washed in saline were performed approximately monthly for a period of 6  
10 months; the first and last immunizations were intravenous and the other immunizations were intraperitoneal. The last injection was performed 3 days prior to fusion. Cell hybridization and selection in HAT medium were performed according to previously described techniques (Kohler *et al.*, *Nature* 256:495 (1975); Fazekas de St. Groth *et al.*, *J. Immunol. Methods* 35:1  
15 (1980); and Lane *et al.*, *J. Immunol. Methods* 72:71 (1984)).

Culture supernatants collected approximately 2 weeks after fusion were screened for antibody activity against MY-10/CD34 antigen in KG-1a cell lysates by immunoblot (Western blot) analysis. Initially, pools of about 10 growth positive hybridoma wells were screened, and individual wells of  
20 antibody positive pools were then screened. Antibody positive wells and were subcloned by limiting dilution (Oi and Hertenberg, in *Selected Methods in Cellular Immunology*, (1980) Mishell and Shigii, eds, p. 351), and clones were screened the same way.

KG-1a cells were solubilized at  $1 \times 10^8$  cells /ml in Laemmli sample  
25 buffer (0.0625 M Tris-HCl, pH 6.8) containing 0.5% Triton X-100 and 2mM PMSF, and centrifuged (30,000 x g, 30 min), and the supernatants were reduced in 50mM DTT, 4% SDS, and 10% glycerol (60 min, 37°C). Electrophoresis was performed on 8-16% pore-gradient, SDS polyacrylamide gels according to the method of Laemmli (*Nature* 227:680, (1970)), as  
30 modified by Jones (in *Selected Methods in Cellular Immunology*, (1980) Mishell and Shigii, eds., pp. 398-440). Proteins were then transferred to

nitrocellulose membranes for immunoblot analysis (Towbin *et al.*, *Proc. Natl. Acad. Sci. USA* 76:4350 (1979) and Burnette, *Anal. Biochem* 112:195 (1981)), using alkaline phosphatase conjugated goat anti-mouse IgG antibody (BioRad labs, Richmond, CA) for detection with BCIP/NBT as substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

The hybridoma clone K6.1 was identified as producing a monoclonal antibody of the IgG2a isotype, as determined with an isotype screening ELISA kit (Zymed Laboratories, S. San Francisco, CA) on immobilized KG-1a cells (Cobbold and Waldmann, *J. Immunol. Methods* 44:125 (1981)). The hybridoma was expanded in roller bottles in IMDM containing fetal calf serum (Hyclone). After supernatant harvesting, the K6.1 antibody was purified by hydroxylapatite chromatography (Stanker *et al.*, *J. Immunol. Methods* 76:157 (1985)), followed by pH-gradient elution from protein A-Sepharose (Ey *et al.*, *Immunochemistry* 15:429 (1978)). The yield of antibody was 40-45  $\mu$ g/liter of supernatant. This was concentrated on ultrafiltration membranes (Amicon YM-10, Danvers, MA), and dialyzed into normal saline. Analysis of antibody purity was performed on 30-40  $\mu$ g reduced and unreduced samples by SDS-polyacrylamide gel electrophoresis under Laemmli conditions, followed by Coomassie blue staining.

For positive immunoselection of CD34<sup>+</sup> cells, all cell washing, incubation and selection steps were performed at 4°C (unless noted otherwise) in 0.2  $\mu$ m sterile filtered "immunoselection washing buffer." The immunoselection washing buffer consisted of Hanks's balanced salt solution containing 12.5 mM HEPES buffer, 1000 units/ml DNase 1 (Calbiochem), and 5% heat-inactivated pooled human AS serum (#34004-1, Pel, Freez Clinical Systems, Brown Deer, WI). The human serum was previously dialyzed extensively (40 volumes x 5 changes) against PBS to remove traces of biotin. This was included as a source of human IgG to saturate Fc receptors and minimize cytophilic binding of the cell specific antibody (i.e., K6.1); for therapeutic immunoselection purposes, it was assumed that

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substitutes such as dialyzed serum from the marrow donor or pharmaceutically approved gamma globulins for injection would be used.

Bone marrow mononuclear cells were washed and adjusted to a concentration of  $50 \times 10^6/\text{ml}$ . Biotinylated-K6.1 antibody was prepared by mixing purified K6.1 with  $\text{NaHCO}_3$  to give a solution containing 3 mg antibody/ml in 0.1 M  $\text{NaHCO}_3$ . Biotin-N-hydroxysuccinimide ester (Calbiochem, La Jolla, CA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 12 mg/ml, and 5.0  $\mu\text{l}$  of this was added to each ml of antibody solution. After 1 hr at room temperature,  $\text{NH}_4\text{HCO}_3$  was added to 50 mM final concentration to stop the reaction. The mixture was then passed through a Sephadex PD-10 column (Pharmacia) equilibrated in phosphate-buffered saline (PBS, 6.7 mM Na phosphate, pH 7.2, 137 mM NaCl) to desalt and exchange the buffer. The biotinylated-K6.1 antibody was added at a ratio of 6-10  $\mu\text{g}/\text{ml}$  of cell suspension and incubated with occasional mixing for 30 min at  $4^\circ\text{C}$ . The cells were then washed by centrifugation 3-4 times, and set to a concentration of  $25 \times 10^6/\text{ml}$ .

DYNABEADS M-450 (DynaI Incorporated, Great Neck, NY) were activated with goat anti-biotin antibody. The number of DYNABEADS used was proportional to the total number of bone marrow mononuclear cells, using a ratio of 1 bead/10 cells. Anti-goat IgG DYNABEADS were washed magnetically 4-5 times with a rare-earth magnet. The beads were then suspended at a concentration of  $1 \times 10^8/\text{ml}$  in washing buffer containing 2.5  $\mu\text{g}/\text{ml}$  affinity purified goat anti-mouse biotin antibody (#SP-3000, Vector Laboratories, Burlingame, CA), mixed vigorously for 30 min at room temperature, and then washed magnetically twice, and resuspended to  $1 \times 10^8/\text{ml}$ . This concentration of anti-biotin was optimized in preliminary titration studies using as an endpoint the kinetics of cell dissociation from the magnetic beads. The binding of anti-goat anti-biotin does not reach equilibrium under these conditions; varying the anti-biotin provided a convenient way to compensate indirectly for different cell surface antigen densities, by controlling the amount of biotin-anti-biotin crosslinking.

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The bone marrow mononuclear cells, containing biotinylated-K6.1 antibody coated target cells, were incubated with the anti-biotin DYNABEADS for 30 min on a rotator (approx. 30 rpm). The magnetic CD34<sup>+</sup> cells were then selected by attraction to a samarium-cobalt magnet, and removal of non-target cells free in suspension. Finally, the magnetic CD34<sup>+</sup> cells were suspended in medium (e.g, IMDM) containing 2.5 mg/ml biotin, put on a rotator for 1-2 hr, and free CD34<sup>+</sup> cells were recovered from magnetically immobilized DYNABEADS. The magnetic separation can be accomplished using the magnetic separation device described in U.S. Patent No. 4,710,472 issued December 1, 1987 to Saur, Reynolds and Black.

Cells isolated by this procedure showed > 99% positive reactivity with a second CD34-specific monoclonal antibody, MY10 (HPCA-2) (Becton Dickinson Immunocytometry Systems, San Jose, CA), by flow cytometric analysis indicating that a highly purified population of cells expressing the CD34 surface membrane antigen was obtained which contained highly enriched hematopoietic stem cells, progenitor cells and essentially no mature blood cells.

Isolated CD34<sup>+</sup> bone marrow cells were cryopreserved (1-5 x 10<sup>6</sup> cells/1 ml vial) and stored under liquid nitrogen prior to experimentation. Before use the CD34<sup>+</sup> cells were thawed using standard techniques.

#### *Processing of CD34<sup>+</sup> Cells for Culture*

Cryopreserved CD34<sup>+</sup> cells were thawed rapidly at 37°C, diluted in a 10 X volume of prewarmed (37°C) complete culture medium. The thawed CD34<sup>+</sup> bone marrow cells were washed twice in complete culture medium, and resuspended at 1 x 10<sup>6</sup> cells/ml. Cell viability was > 99% as determined by trypan blue dye exclusion (Coligan *et al.*, *Current Protocols in Immunology* (1992), Greene Publishing and Wiley-Interscience, New York).

At the start of experimentation, a sample of CD34<sup>+</sup> cells was cultured to determine the number and type of hematopoietic colony forming cells

(CFC) using a methylcellulose colony forming assay (Meisenberg *et al.*, *Blood* 79:2267 (1992)). Briefly, purified CD34<sup>+</sup> bone marrow cells and nonadherent hematopoietic cells from harvested cultures were cultured in 35 mm Lux suspension culture dishes (Miles Laboratories, Naperville, IL) using a modification of the technique previously described (Meisenberg *et al.*, *Blood* 79:2267 (1992)). One milliliter of culture consisted of 5-500 x 10<sup>2</sup> bone marrow cells, IMDM medium (Quality Biologicals, Rockville, MD), 1% methylcellulose, 30% heat-inactivated fetal calf serum (FCS), 2 U/ml tissue culture grade erythropoietin (Amgen, Thousand Oaks, CA), 2 ng/ml GM-CSF, 10 ng/ml IL-3 (R&D Systems, Minneapolis, MN) and 5% conditioned medium from the bladder carcinoma cell line 5637 (ATCC, Rockville, MD). The conditioned medium from cell line 5637 was used as a source of colony stimulating activity. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. On day 14 of incubation, cultures were evaluated to determine the number of colonies (>50 cells) which had developed. At day 14, aggregates of hemoglobin containing cells were recognized as BFU-E; aggregates of granulocytes and/or macrophages and/or megakaryocytes as CFU-MIX; and aggregates containing only granulocytes and macrophages as CFU-GM. Megakaryocyte colonies (CFU-Mk) were confirmed based upon established morphological criteria (Williams and Levin, *Br. J. Hematol.* 52:173 (1982)).

#### ***Endothelial Cell Culture Conditions***

Porcine brain microvascular endothelial cells (PMVEC) were isolated and grown as previously described (Robinson *et al.*, *In Vitro Cell. Dev. Biol.* 26:169 (1990)). The phenotypic and growth properties of these cells have been extensively characterized (Robinson *et al.*, *In Vitro Cell. Dev. Biol.* 26:169 (1990) and Robinson *et al.*, *Blood* 77:294 (1991)).

Briefly, PMVECs were isolated from the brains of 4 to 6 month old Yucatan minipigs. The brains were collected aseptically, immersed in 10%

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povidone-iodine (Sherwood Pharmaceutical Co., Rahway, NJ) for 2 min and washed 5-6 times with Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY) to remove the residual iodine. Gray matter of the cortices was aspirated through a Pasteur pipette, centrifuged for 10 min at 500 g (room temperature), resuspended in HBSS and homogenized in a 40 ml dounce homogenizer. The homogenate was centrifuged, resuspended and subsequently sieved through sterile nylon fabric of 149, 76, and 20 micro mesh size. The retained microvessels were resuspended in 6 ml HBSS and spun through discontinuous Ficoll-Paque gradients (33%-67% and 67%-75%) (Pharmacia Inc., Piscataway, NJ). The pelleted microvessels were resuspended in 2 ml HBSS and 2 ml collagenase (1 mg/ml) (Worthington Biomedical, Freehold, NJ) was added. After 2 mins, the microvessels were washed with HBSS and plated in 16-mm wells coated with fibronectin (Pierce Chemical, Rockport, IL) in M199 media (Quality Biological Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum, 500 micrograms/ml sodium heparin (Sigma Chemicals, St. Louis, MO), and 2-10 uL/ml retinal-derived growth factor (see Robinson *et al.*, *In Vitro Cell. Dev. Biol.* 26:169 (1990)). The cultures were then grown at 37°C in air with 5% CO<sub>2</sub>. After 7 days the cells were subcultured and subcloned. After subcloning, the PMVEC cell lines were grown in complete culture medium.

PMVEC were fed weekly with complete medium. When confluent, PMVEC were washed with PBS, trypsinized (0.25 mg trypsin/mL, 5mM EDTA, 37°C, 10 minutes, Sigma, St. Louis, MO) and subcultured in a ratio of 1:5 into either 75 cm<sup>2</sup> flasks or a cellular concentration of  $1 \times 10^5$  cells/well in gelatin-coated 6-well, tissue culture plates (Costar, Cambridge, MA) containing 3 mL of complete culture medium supplemented with an additional 10% FCS. After 48-72 hr, the adherent PMVEC monolayers (70-80% confluent) were washed twice with complete culture medium to remove nonadherent PMVEC and the culture medium was replaced with 5 mL of complete cell culture medium.

### *Long-Term Marrow Cultures in Tissue Culture Flasks*

Purified CD34<sup>+</sup> bone marrow cells ( $5 \times 10^5$  cells/flask) were inoculated in 75 cm<sup>2</sup> flasks containing confluent PMVEC monolayers, and 15 mL of complete culture medium supplemented with GM-CSF (2 ng/mL), IL-3 (10 ng/mL), SCF (100 ng/mL) and IL-6 (10 ng/mL). All cultures were maintained at 37°C in a humidified atmosphere at 5% CO<sub>2</sub> in air. After 7 days, and subsequently at weekly intervals, all nonadherent cells were removed and counted, and  $2 \times 10^6$  nonadherent cells were reseeded onto fresh PMVEC monolayer cultures containing fresh medium supplemented with growth factors. Nonadherent cells were immunophenotyped for the CD34 and CD38 cell surface antigens (see below).

### *Artificial Capillary System*

The artificial capillary system (CELLMAX Quad, Cellco, Inc., Germantown, MD) used in this study consisted of a 2 x 13 cm cylindrical cartridge containing hollow capillary fibers made of polypropylene, silicone rubber tubing which serves as a gas exchanger to maintain the proper pH and pO<sub>2</sub>, a 125 ml medium bottle which serves as a reservoir, and an external pump. Prior to inoculating the ACS cartridge with endothelial and purified CD34<sup>+</sup> bone marrow cells (see below), the polypropylene capillaries were coated with a recombinant adhesion protein having the amino acid sequence shown in Figure 10 (sold under the trademark PRONECTIN™ F, Protein Polymer Technologies, Inc., San Diego, CA.), as described below.

The ACS cartridge was filled with 95% ethanol for 30 mins, rinsed with deionized water and the procedure repeated. The cartridge was then autoclaved for 45 mins at 250°F. After cooling, either a 1% or 25% solution of the recombinant adhesion protein (in phosphate buffered saline (PBS) at 37°C) was added, filling the extra capillary space (ECS) and fiber lumen to coat the polypropylene fibers. After one hour, the cartridge was thoroughly

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washed for approximately 30 mins with 0.3 liters of deionized water at a flow rate of 10 mL/min followed by autoclaving for 45 mins at 250°F. The cartridge was then inserted into sterile flow paths and mounted into pump stations on the CELLMAX Quad (Cellco, Inc., Germantown, MD.). The cartridge was then flushed thrice with 120 mL of complete culture medium over a 48 hour period before replacing the growth medium reservoir bottle with fresh pre-warmed (37°C) complete culture medium. All operation were performed using sterile technique under a laminar flow hood.

During culture (see below), complete culture medium was drawn from the reservoir, pumped through the gas exchange tubing and lumen of the capillaries, and then returned to the reservoir for subsequent recirculation. Nutrients and oxygen in the medium diffused through the capillary walls into the 7 ml ECS, and cell metabolites diffused back from the ECS into the perfusate. The flow rate of the pump was maintained at approximately 50 ml/min over the duration of the culture and the system was kept at 37°C in 5% CO<sub>2</sub> in a humidified incubator. The polypropylene capillary modules had a surface area of approximately 0.04 m<sup>2</sup> and had a pore size of 0.5 μ in the capillary walls.

#### *Large-Scale ACS cell culture of purified CD34<sup>+</sup> bone marrow cells*

PMVEC (1 x 10<sup>8</sup> cells) were injected into the ECS and dispersed over the hollow capillaries by flushing culture medium back and forth through the two sampling side-ports using one 10 ml syringe filled with complete culture medium plus cells and another empty 10 ml syringe. After 72 hrs of culture, nonadherent PMVEC which had not settled onto the outside of the hollow capillaries were harvested from the ECS by purging with pre-warmed complete culture medium through the two sample side-ports.

Purified CD34<sup>+</sup> bone marrow cells (1-4 x 10<sup>6</sup> cells/cartridge) were injected into the ECS through the two side sampling ports and the spent culture medium replaced with 120 mL of hematopoietic cell growth medium

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consisting of Iscoves (IMDM) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 2 ng/mL GM-CSF, 10 ng/mL IL-3, 120 ng/mL SCF, and 10 ng/mL IL-6.

After 7 days of culture, and subsequently at weekly intervals, all the nonadherent cells were harvested by flushing from the ECS. 75% of the harvested cells were pelleted during a 400 g centrifugation for 10 min at 18°C, resuspended in complete culture medium, and enumerated using a hemacytometer and trypan blue dye. 25% of the harvested cells were returned to the ACS for further expansion. Nonadherent cells were immunophenotyped for the CD34 and CD38 cell surface antigens (see below).

Cell harvest and media exchange procedures required approximately 5 min to complete during which time the cartridge system was not perfused. The growth medium (~pH 7.3) was monitored for changes in pH and was changed approximately every 2-3 days when its pH decreased to pH 7.0.

#### ***Immunophenotype of Cultured Bone Marrow Cells by Immunofluorescence Staining***

Simultaneous two-color cytometric analysis of cultured nonadherent cells were performed at selected intervals of culture to measure the expression of CD34 and CD38 cell surface antigens. Briefly, nonadherent cells which were cultured in flask culture and using the ACS (as described above) were harvested, washed twice in complete culture medium, and resuspended in PBS supplemented with 2% (wt/vol) bovine serum albumin (BSA) and 0.1% sodium azide (staining medium). Nonadherent cells were first incubated for 30 mins with saturating concentrations of anti-CD34 monoclonal antibody (K6.1 MoAb). After two washes with staining medium, cells were incubated for an additional 30 minutes with biotinylated goat anti-mouse (IgG1). After a second series of two washes with staining medium, FITC-conjugated CD38 (OKT10 MoAb) and APC-streptavidine were added together and incubated for an additional 30 minutes. Finally, cells were washed twice with staining

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medium and fixed with 1% paraformaldehyde. Each incubation step was done in the dark, at 4°C, and cells stained with the appropriate conjugated isotype control antibodies. At least 10,000 events were collected in listmode on a Coulter Elite (Coulter, Hialeah, FL) flow cytometer.

5      ***Methylcellulose Assay for Colony Forming Unit-Granulocyte/Macrophage (CFU-GM)***

Purified CD34<sup>+</sup> bone marrow cells and cultured nonadherent hematopoietic cells harvested from the ACS cartridge were cultured in 35 mm Lux suspension culture dishes (Miles Laboratories, Naperville, IL) using a  
10      modification of the technique as previously described (Meisenberg *et al.*, *Blood* 79:2267 (1992)). One milliliter of culture consisted of 5-500 x 10<sup>2</sup> bone marrow cells, Iscoves IMDM medium (Quality Biologicals, Rockville, MD), 1% methylcellulose, 30% FCS, 2 U/mL tissue culture grade erythropoietin (Amgen, Thousand Oaks, CA), 2 ng/mL GM-CSF, 10 ng/mL IL-3, 5 ng/mL  
15      IL-6 (R&D Systems, Minneapolis, MN) and 5% conditioned medium from the bladder carcinoma cell line 5637 (5 X concentrate) as a source of colony stimulating activity. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. On day 14 of incubation, cultures were evaluated to determine the number of colonies (>50 cells) developed in  
20      methylcellulose. At day 14, aggregates of hemoglobin containing cells were recognized as BFU-E, granulocyte-macrophage colonies as CFU-GM and aggregates of hemoglobin cells containing at least granulocytes and/or macrophages and/or megakaryocytes as CFU-Mix. Megakaryocyte colonies (CFU-MK) were confirmed based upon established morphological criteria  
25      (Williams and Levin, *Br J Hematol* 52:173 (1982)). Three dishes were set up for each individual data point per experiment.

## Results

### *Comparison of Flask vs. ACS Culture of Hematopoietic Cells*

Purified human bone marrow CD34<sup>+</sup> cells were cultured on confluent PMVEC monolayers in 75 cm<sup>2</sup> tissue culture flasks and on PMVEC immobilized onto the surface of capillaries within the ECS of an ACS cartridge. A total of  $1 \times 10^6$  CD34<sup>+</sup> cells ( $5 \times 10^5$  cells/flask) were inoculated into two flask cultures and a total of  $3 \times 10^6$  CD34<sup>+</sup> cells were inoculated into the ECS. Each flask and the ECS were inoculated with  $5 \times 10^5$  and  $1 \times 10^8$  PMVEC respectively, one week prior to the CD34<sup>+</sup> inoculation. The details of the flasks and ACS cultures are discussed above in the Materials and Methods section.

After seven days of culture, non-adherent cells from the flasks and ACS cultures were harvested and subject to immunophenotyping with monoclonal antibodies against CD34 and CD38 cell surface antigens. The results are shown in Figure 1. The purity of the starting CD34<sup>+</sup> cell population was approximately 85% CD34<sup>+</sup>CD38<sup>+</sup> and approximately 15% CD34<sup>+</sup>CD38<sup>-</sup>. After 7 days of culture in the ACS, there was a 15-fold expansion of CD34<sup>+</sup> cells. Moreover, after 7 days of culture in the ACS, the absolute number of CD34<sup>+</sup>CD38<sup>+</sup> cells and CD34<sup>+</sup>CD38<sup>-</sup> cells increased 6.2-fold and 70.5-fold, respectively. However, in flask culture, only a 11-fold expansion of CD34<sup>+</sup> cells occurred after 7 days of expansion. Moreover, after 7 days of flask culture, the absolute number of CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> cells increased 6.7-fold and 35.7-fold, respectively.

These data indicate that (1) the ACS is capable of substantial expansion of CD34<sup>+</sup> cells; (2) this expansion is greater than that achieved using the same system (i.e., culture on PMVEC support cells) in tissue culture flasks; and (3) the ACS is superior to the flask culture in expanding the primitive CD34<sup>+</sup>CD38<sup>-</sup> HSC. Thus, the ACS system is better able to support primitive HSC expansion without differentiation and depletion.

### ***Long Term Hematopoietic Cell Production in ACS Culture***

1 x 10<sup>8</sup> PMVEC were inoculated into the ECS of an ACS cartridge. After one week, 4 x 10<sup>6</sup> human bone marrow CD34<sup>+</sup> cells were inoculated into the ECS. The details of the ACS culture are described above in the Materials and Methods section. The culture was continuously maintained for 8 days. The total number of nonadherent cells produced within the ACS was enumerated at weekly intervals where approximately 75% of cells contained in the ECS were harvested. Manual hemacytometer cell counts were performed using trypan blue exclusion dye. Cumulative nonadherent hematopoietic cell yield is shown in Figure 2. These data show that a total of 8 x 10<sup>12</sup> hematopoietic cells can be generated over 78 days from a starting population of 4 x 10<sup>6</sup> CD34<sup>+</sup> cells; a 2 million-fold expansion of hematopoietic cells. These results demonstrate that the ACS/PMVEC culture system supports long-term hematopoiesis.

### ***Long Term CD34<sup>+</sup> HSC Production in ACS Culture***

1 x 10<sup>8</sup> PMVEC were inoculated into the ECS of an ACS cartridge. After one week, 4 x 10<sup>6</sup> human bone marrow CD34<sup>+</sup> cells were inoculated into the ECS. The details of the ACS culture are described above in the Materials and Methods section. The culture was continuously maintained for 35 days. The total number of CD34<sup>+</sup> cells produced was enumerated weekly using the anti-CD34 MoAb K6.1 and flow cytometry techniques as described above in the Materials and Methods section. Cumulative CD34<sup>+</sup> cell yield is shown in Figure 3.

These data demonstrate a 150-fold expansion of CD34<sup>+</sup> cells over 35 days of ACS culture. Thus, the ACS/PMVEC system supports the proliferation and expansion of the CD34<sup>+</sup> bone marrow stem cell pool for at least five weeks.

### ***Long Term CFU-GM Production in ACS Culture***

1 x 10<sup>8</sup> PMVEC were inoculated into the ECS of an ACS cartridge. After one week, 4 x 10<sup>6</sup> human bone marrow CD34<sup>+</sup> cells were inoculated into the ECS. The details of the ACS culture are described above in the Materials and Methods section. The culture was continuously maintained for 35 days. The total number of granulocyte-macrophage colony-forming units (CFU-GM) was enumerated weekly using the methylcellulose assay described above. Cumulative CFU-GM yield is shown in Figure 4.

These data demonstrate a 1609-fold expansion of CFU-GM over 28 days of culture. More importantly, 1.0 x 10<sup>7</sup> CFU-GM's, the number necessary to clinically transplant a patient, can be generated from a single ACS/PMVEC system within 14 days of culture.

Moreover, after 28 days of ACS/PMVEC culture, the methylcellulose clonogenic cell assays revealed 77-fold expansion of CFU-Blast, 4222-fold expansion of CFU-Mix, 388-fold expansion of CFU-Mk, and 454-fold expansion of BFU-E when compared to pre-expansion levels.

### ***Example 2***

#### ***Co-cultivation of PMVEC intraluminally with CD34<sup>+</sup> Cells in the ECS***

##### ***Nonadherent Hematopoietic Cell Production***

Prior to inoculating the ACS cartridge with endothelial and CD34<sup>+</sup> bone marrow cells (see below), the ACS polypropylene capillaries were coated with the recombinant adhesion protein (PRONECTIN™ F, Protein Polymer Technologies, Inc., San Diego, CA.) as described in Example 1.

3 x 10<sup>7</sup> PMVEC (prepared as described in Example 1) were inoculated into the lumen of the polypropylene capillaries via the endport. The cartridge

containing the capillaries was then incubated at 37°C and rotated every 15-20 mins for 2 hours to allow the PMVEC to adhere to the inner wall of the capillaries. This was followed by incubation at 37°C for approximately 13 hours. The cartridge was then perfused with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FBS, 1% penicillin/streptomycin and 2mM L-glutamine at a flow rate of 0.40 ml/min.

CD34<sup>+</sup> cells were enriched from a 10 ml iliac crest bone marrow aspirate from a human patient using the Cellpro Cephate LC34 Cell Separation System (Cellpro, Inc.). Enriched CD34<sup>+</sup> cells ( $1 \times 10^6$  cells/cartridge) were injected into the ECS of the cartridge and the cartridge was perfused with IMDM supplemented with 5% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 1 ng/ml GM-CSF, 5 ng/ml IL-3, 5 ng/ml IL-6, and 120 ng/ml SCF. The volume of medium in the reservoir bottle was 100 ml.

Periodically during ACS culture (at days 5, 7, 8, 10, 12, and 13), spent medium was removed from the reservoir bottle and replace with a corresponding amount of fresh medium.

After 7 days of co-culturing the PMVEC (immobilized in the capillary lumen) and enriched CD34<sup>+</sup> HSC (located in the ECS), the nonadherent hematopoietic cell population was harvested from the ECS by vigorous flushing from the two sideports. A total of  $6.1 \times 10^6$  viable nonadherent cells were counted using a hemacytometer and trypan blue viability dye.  $2.8 \times 10^6$  of the nonadherent cells were reinoculated into the ECS and the culture continued. After an additional 7 days of culture, nonadherent hematopoietic cells were again harvested from the ECS by vigorous flushing from the two sideports.  $1.05 \times 10^7$  harvested nonadherent cells were enumerated and cumulative nonadherent cell yield is shown in Figure 5. These data demonstrate that a 22.8-fold expansion of nonadherent hematopoietic cells can be generated in the ECS within 14 days of co-culture with the PMVEC immobilized in the capillary lumen.

### ***Colony Forming Cell (CFC) Production***

Enriched CD34<sup>+</sup> cells taken prior to culture and nonadherent cells harvested after 14 days of culture were assayed for the total number of colony-forming cells (CFC) using a clonogenic assay medium supplied by Terry Fox Labs (Vancouver, Canada, Kit No. HCC-4330). At days 0 and 14 of culture, the total number of CFC per 10,000 nonadherent cells plated in the Terry Fox medium was scored. The cumulative total CFC yield is shown in **Figure 6**. These data demonstrate that PMVEC cultured intraluminally support a 80.6-fold expansion of CFC progenitor cells when the CD34<sup>+</sup> cells were co-cultured within the ECS for 14 days.

### ***Lactate Production***

The lactic acid concentration in the ECS during culture was determined using a YSI glucose/lactate automated analyzer (Yellow Springs Instrument Co., Inc.). In particular, the daily lactate production was calculated by dividing the change in total lactate content by the days between measurements, plotting the rate of lactate production, and determining the doubling time of lactate production based on logarithmic growth rate. The results are shown in **Figure 7**.

### ***Example 3***

#### ***The Beneficial Effects of Coating the ACS Capillaries with an Adhesion Protein***

##### ***Experimental Protocol***

5 Human iliac crest bone marrow cells were harvested from a patient after informed consent. The cells were washed from marrow filtration screens and subject to Ficoll-Hypaque (specific gravity 1.077 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ) density gradient separations. Mononuclear cells from the interface band were collected after centrifugation at 300g for 25 min  
10 at 25°C. The cells were then resuspended in Iscove's modified Dulbecco's medium supplemented with 12.5% FCS + 12.5% horse serum + 2 mM L-glutamine + 100 U/ml Penicillin/Streptomycin.

ACS polypropylene capillaries were coated with the recombinant adhesion protein (PRONECTIN™ F, Protein Polymer Technologies, San  
15 Diego, Ca. 92121) as described above in Example 1. Briefly, the capillaries were washed with ethanol and then with deionized water and the process repeated. The capillaries were then autoclaved twice for 45 min at 250°F and then treated with the adhesion protein (1% dilution of stock adhesion protein in phosphate buffered saline (PBS)) under sterile conditions or treated with  
20 PBS only. ACS cartridges containing the capillaries (with and without the adhesion protein coating) were inserted into sterile flow paths and mounted into pump stations on the CELLMAX Quad (Cellco, Inc., Germantown, MD.). The cartridge was then perfused with Iscove's medium containing 10 mg/ml bovine serum albumin (BSA), 1% penicillin/streptomycin solution for  
25 two days. The perfusion medium was replaced with 50 ml of hematopoietic growth medium containing fresh Iscove's medium supplemented with glutamine (1%), penicillin/streptomycin (1%), fetal calf serum (12.5%), horse

serum (12.5%), IL-3 (4 ng/ml), IL-6 (5 ng/ml), GM-CSF (1 ng/ml) and stem cell factor (2 ng/ml).

33 x 10<sup>6</sup> bone marrow mononuclear cells were injected into the ECS of cartridges containing capillaries either with or without the adhesion protein coating. After days 4, 6, 8, 9, 10 and 11 of culture, 25 ml aliquots of fresh hematopoietic growth medium were added to the reservoir bottle. After 12 days of culture, nonadherent cells were harvested from the ECS and the number of colony-forming cells (CFU-GM and BFU-E) was determined using a methylcellulose clonogenic cell assay kit (Terry Fox Labs, Vancouver, Canada).

Glucose utilization was measured by taking 2 ml aliquots of medium from the ACS reservoir on days 4 and 6 and performing utilization analysis on a YSI glucose/lactate analyzer (Yellow Springs Instrument Co., Inc.).

### *Results*

After 12 days of culture, the number of CFU-GM + BFU-E clonogenic hematopoietic progenitor cells per 200,000 harvested nonadherent cells was measured using a methylcellulose clonogenic cell assay that supports multilineage colony-formation. The results are shown in Figure 8. These data demonstrate that the adhesion protein-coated capillaries enhance hematopoietic progenitor cell growth 2.8-fold in comparison to capillaries without the adhesion protein coating.

After days 4 and 6 of culture, aliquots of culture medium (2 ml) were removed from the ACS reservoir and analyzed on a YSI glucose/lactate analyzer (Yellow Springs Instrument Co., Inc.) for glucose utilization. Under the culture conditions, the glucose utilization assay is primarily a measure of stromal cell activity. Glucose values were measured on day 4 and day 6 and the amount of glucose consumed per 2 days was calculated and divided by 2 to give the glucose consumption per 24 hr. Glucose consumption (gm/24 hours) is shown in Figure 9. These data demonstrate that adhesion protein-

coated capillaries provide an approximately 1.8-fold increase in stromal cell activity as compared to capillaries without the adhesion protein coating.

#### ***Example 4***

### ***Consolidation Therapy with Autologous Ex Vivo Expanded Hematopoietic Cells***

#### ***Outpatient Chemotherapy of Malignant Breast Cancer Patients***

Outpatient induction chemotherapy is performed using the multidrug, dose-intense, 16-week regimen described in Kennedy *et al.*, *J Natl Cancer Inst* 83 (13):921 (1991); Abeloff *et al.*, *J Natl Cancer Inst* 82:570 (1990); and Beveridge *et al.*, *Proc ASCO* 7:13 (1988). Briefly, malignant breast cancer patients receive 100 mg/m<sup>2</sup> cyclophosphamide orally on days 1 through 7, 40 mg/m<sup>2</sup> doxorubicin intravenously (IV) on day 1, 100 mg/m<sup>2</sup> methotrexate IV on day 1 with 10 mg/m<sup>2</sup> leucovorin rescue orally every 6 hours for six doses beginning on day 2, 1 mg vincristine IV on day 1, and 600 mg/m<sup>2</sup> fluorouracil (5-FU) IV for 2 hours at hour 20. On days 8 and 9, patients receive 300 mg/m<sup>2</sup> 5-FU IV daily by continuous infusion through an indwelling venous access device. Patients receive a maximum of eight 2-week cycles.

After completion of outpatient therapy, patients showing a complete or partial response by standard ECOG criteria are candidates for further therapy. These patients are then subject to bone marrow harvest and high-dose chemotherapy 4-6 weeks after the outpatient treatment as described below.

#### ***Harvest and Ex-Vivo Expansion of Autologous Bone Marrow***

Bone marrow is harvested from the posterior iliac crest from the patients as described in Kennedy *et al.*, *J Natl Cancer Inst* 83 (13):921 (1991).

After harvesting, the bone marrow cells are subject to purging with 4-hydroperoxycyclophosphamide (4-HC) at a dose of 100 ug/mL at 37°C for 30 min to remove contaminating viable tumor cells. After purging, the cells are rapidly cooled to 4°C and resuspended in 85% Tissue Culture 199 (GIBCO Laboratories, Grand Island, NY), 5% autologous plasma, and 10% dimethyl sulfoxide.

CD34<sup>+</sup> cells are purified from the marrow cells and co-cultured with PMVEC in an ACS cartridge as described in Example 1. After two weeks of ACS culture, nonadherent hematopoietic cells (including at least 10<sup>7</sup> CD34<sup>+</sup> cells and 10<sup>7</sup> CFU-GM) are harvested from the ECS by flushing with culture medium as described in Example 1.

After the marrow harvest, the patients are again subject to chemotherapy as described in Kennedy *et al.*, *J Natl Cancer Inst* 83 (13):921 (1991). Briefly, patients receive continuous infusion of 1.5 g/m<sup>2</sup> cyclophosphamide and 200 mg/m<sup>2</sup> thiotepa daily for 4 days. Four days after completion of chemotherapy, the harvested autologous hematopoietic cells recovered from ACS culture are reinfused into the patients.

### ***Example 5***

#### ***Transduction of Hematopoietic Cells with Packaged Retroviral Vectors***

##### ***Preparation of Packaged LASN***

LASN is a retrovirus vector containing the ADA and *Neo* resistance genes. High concentrations of packaged LASN retrovirus particles are produced in the supernatant of LASN-producing PA-317 packaging cells (ATCC Accession No. CRL 9078) as described in Hock *et al.*, *Blood* 74:876-

881 (1989). 5 ml of supernatant from the packaging cells is filtered through a POLYDISC™ AS filter (Whatman Ltd., Maidstone, England) and collected for transduction.

#### ***Transduction of Hematopoietic Cells with Packaged LASN***

5            Bone marrow is harvested from the posterior iliac crest from a human subject using the technique described in Kennedy *et al.*, *J Natl Cancer Inst* 83 (13):921 (1991). After harvesting, the CD34<sup>+</sup> HSC are purified from the marrow cells and co-cultured with PMVEC in an ACS cartridge as described in Example 1. After one week of culture, non-adherent cells are removed by  
10            gently shaking (or flushing) the ACS cartridge. About 20% of the cells are separated to serve as non-transduced controls. The remaining cells are pelleted and resuspended in 5 ml of the LASN filtrate described above. The resuspension, containing packaged LASN and nonadherent hematopoietic cells, is reinoculated into the ACS cartridge and the culture continued. After two  
15            days, the transduction procedure is repeated with a second volume of LASN filtrate. After two more days, the transduction procedure is again repeated. Before each transduction procedure, samples of expanded hematopoietic cells are removed from the ACS for analysis. The culture is terminated after about eight days. Transduced hematopoietic stem cells may be recovered both in the  
20            nonadherent cell population and bound to the endothelial layer.

#### ***Detection of LASN in Nonadherent and Bound Hematopoietic Cells***

            The presence of LASN in nonadherent and bound (to the endothelial layer) hematopoietic cells is confirmed by the polymerase chain reaction (PCR). PCR analysis is carried out using GeneAmp<sup>®</sup> reagents and DNA  
25            Thermal Cycler (Perkin Elmer Cetus, Emeryville, CA). DNA is isolated from the transduced and non-transduced hematopoietic cells according to conventional techniques. PCR is initiated with 1-2 µg of genomic DNA using

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primers flanking the *Neo* resistance gene which is contained in LASN. The DNA sequences of the primers are as follows:

CAAGATGGATTGCACGCAGG

CCCGCTCAGAAGAACTCGTC

5 The reaction mixture is heated at 94°C for 2 min, annealed at 56°C for 2 min, and extended at 72°C for 3 min in the DNA Thermal Cycler for 30 cycles. The products of the reaction are loaded and run on a gel and probed with a *Neo*-resistance gene specific probe.

10 It will be appreciated to those skilled in the art that the invention can be performed within a wide range of equivalent parameters of composition, concentrations, modes of administration, and conditions without departing from the spirit or scope of the invention or any embodiment thereof.

The disclosure of all references, patent applications and patents recited herein are hereby incorporated by reference.

(1) GENERAL INFORMATION:

- (i) APPLICANT: United States of America as represented by the Secretary of the Navy and Cellco, Inc.
- (ii) INVENTORS: Davis, Thomas A.  
Lee, Kelvin P.  
Kidwell, William R.
- (iii) TITLE OF INVENTION: Hematopoietic Cell Expansion and Transplantation Methods
- (iv) NUMBER OF SEQUENCES: 3
- (v) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Sterne, Kessler, Goldstein & Fox
  - (B) STREET: 1100 New York Avenue, Suite 600
  - (C) CITY: Washington
  - (D) STATE: D.C.
  - (E) COUNTRY: USA
  - (F) ZIP: 20005
- (vi) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vii) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: (to be assigned)
  - (B) FILING DATE: 20 January 1995
  - (C) CLASSIFICATION:
- (viii) PRIORITY APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/184,140
  - (B) FILING DATE: 21 JANUARY 1994
  - (C) CLASSIFICATION:
- (ix) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Goldstein, Jorge A.
  - (B) REGISTRATION NUMBER: 29,021
  - (C) REFERENCE/DOCKET NUMBER: 1444.015PC00
- (x) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (202) 371-2600
  - (B) TELEFAX: (202) 371-2540

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 980 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: both

Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
1 5 10 15  
Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
20 25 30  
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35 40 45  
Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser

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Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 50 55 60  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 65 70 75 80  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 85 90 95  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Val Thr Gly  
 100 105 110  
 Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser  
 115 120 125  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 130 135 140  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 145 150 155 160  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 165 170 175  
 Gly Ala Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly  
 180 185 190  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 195 200 205  
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 210 215 220  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 225 230 235 240  
 Gly Ala Gly Ala Gly Ser Gly Ala Ala Val Thr Gly Arg Gly Asp Ser  
 245 250 255  
 Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 260 265 270  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 275 280 285  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 290 295 300  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Val  
 305 310 315 320  
 Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala  
 325 330 335  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 340 345 350  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 355 360 365  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 370 375 380  
 Gly Ser Gly Ala Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala  
 385 390 395 400  
 Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 405 410 415  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 420 425 430

-51-

Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 435 440 445  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Val Thr Gly Arg Gly  
 450 455 460  
 Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala  
 465 470 475 480  
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 485 490 495  
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 515 520 525  
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 690 695 700  
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 770 775 780  
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 785 790 795 800  
 Gly Ala Gly Ala Gly Ser Gly Ala Ala Val Thr Gly Arg Gly Asp Ser  
 805 810 815

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Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
                     820                    825                    830  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
                     835                    840                    845  
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 Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala  
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                     915                    920                    925  
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                     945                    950                    955                    960  
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 Gly Ala Gly Ser  
                     980

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 16 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Ala Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly  
 1                    5                    10                    15

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ala Gly Ala Gly Ser  
 1                    5

***We Claim:***

1. A method for transplanting *ex vivo* expanded hematopoietic cells, including hematopoietic stem and progenitor cells, into a patient comprising:

- 5 (a) inoculating endothelial cells capable of supporting expansion of the hematopoietic cells into an artificial capillary system (ACS) cartridge;
- (b) inoculating CD34<sup>+</sup> cells into said ACS cartridge;
- (c) perfusing said ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of  
10 the hematopoietic cells;
- (d) culturing said CD34<sup>+</sup> cells in said ACS cartridge for a sufficient amount of time to achieve expansion of a therapeutically effective number of hematopoietic cells;
- (e) harvesting cultured cells from said ACS cartridge; and  
15 (f) transplanting said cells into the patient.

2. The method of claim 1 wherein said harvested cells include a sufficient number of CD34<sup>+</sup>CD38<sup>-</sup> hematopoietic stem cells (HSC) to achieve long-term reconstitution of the hematopoietic system.

3. The method of claim 1 wherein said harvested cells include a  
20 sufficient number of committed hematopoietic progenitor cells to achieve short-term reconstitution of the hematopoietic system.

4. The method of claim 1 wherein said endothelial cells are immobilized on the outer capillary wall of said ACS cartridge.

5. The method of claim 1 wherein said endothelial cells are  
25 immobilized on the inner capillary wall of said ACS cartridge.

6. The method of claim 1 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as an enriched population of CD34<sup>+</sup> cells.

7. The method of claim 1 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as a subpopulation of CD34<sup>+</sup> cells included within a mixed cell population.

8. The method of claim 1 wherein the patient is suffering from a malignancy.

9. The method of claim 8 wherein the CD34<sup>+</sup> cells are enriched from the patient's bone marrow cells.

10. The method of claim 8 wherein said CD34<sup>+</sup> cells are purged of contaminating tumor cells prior to inoculation.

11. A method for the *ex vivo* expansion of hematopoietic cells, including hematopoietic stem and progenitor cells, comprising:

(a) inoculating endothelial cells capable of supporting expansion of the hematopoietic cells into an artificial capillary system (ACS) cartridge;

(b) inoculating CD34<sup>+</sup> cells into said ACS cartridge;

(c) perfusing said ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of the hematopoietic cells;

(d) culturing said CD34<sup>+</sup> cells in said ACS cartridge for a sufficient amount of time to achieve hematopoietic cell expansion.

12. The method of claim 11 further comprising:

(e) harvesting cultured cells from said ACS cartridge.

13. The method of claim 12 further comprising:

(f) detecting the presence of expanded CD34<sup>+</sup> cells.

14. The method of claim 12 further comprising:

(f) detecting the presence of expanded CD34<sup>+</sup>CD38<sup>-</sup> hematopoietic stem cells (HSC).

15. The method of claim 12 further comprising:

(f) detecting the presence of expanded CFU-GM hematopoietic progenitor cells.

16. The method of claim 13 wherein said cells are detected by phenotype analysis.

17. The method of claim 14 wherein said cells are detected by phenotype analysis.

18. The method of claim 15 wherein said cells are detected by a colony forming assay.

19. The method of claim 11 wherein culturing occurs for a sufficient amount of time to achieve long term hematopoiesis.

20. The method of claim 11 wherein said endothelial cells are immobilized on the outer capillary wall of said ACS cartridge.

21. The method of claim 11 wherein said endothelial cells are immobilized on the inner capillary wall of said ACS cartridge.

22. The method of claim 11 wherein said endothelial cells are porcine.

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23. The method of claim 22 wherein said endothelial cells are PMVEC.

24. The method of claim 11 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as an enriched population of CD34<sup>+</sup> cells.

5 25. The method of claim 11 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as a subpopulation of CD34<sup>+</sup> cells included within a mixed cell population.

26. The method of claim 24 wherein said cells are enriched using an antibody specific for the CD34 cell surface antigen.

10 27. The method of claim 11 wherein said hematopoietic growth factor is selected from the group consisting of IL-1, IL-1 $\alpha$ , IL-1 $\beta$ , G-CSF, GM-CSF, IL-3, IL-6, IL-11, erythropoietin, LIF, PIXY-321 and SCF.

28. The method of claim 27 wherein said growth factors are GM-CSF, IL-3, SCF and IL-6.

15 29. The method of claim 24 wherein said CD34<sup>+</sup> cells are enriched from a source of cells selected from the group consisting of bone marrow cells, peripheral blood cells, and umbilical cord blood cells.

20 30. The method of claim 25 wherein said subpopulation of CD34<sup>+</sup> cells are included within a mixed cell population selected from the group consisting of bone marrow cells, peripheral blood cells, and umbilical cord blood cells.

31. The method of claim 11 wherein the capillaries of said ACS cartridge are coated with a suitable coating reagent.

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32. The method of claim 31 wherein said coating reagent is an adhesion protein having an amino acid sequence substantially identical to that shown in Figure 10 (SEQ ID NO. 1).

33. An artificial capillary system (ACS) comprising:

- 5 (a) an ACS cartridge;
- (b) culture medium containing at least one hematopoietic growth factor;
- (c) CD34<sup>+</sup> cells; and
- 10 (d) endothelial cells capable of supporting expansion of hematopoietic cells, including hematopoietic stem and progenitor cells;
- wherein said culture medium, said CD34<sup>+</sup> cells, and said endothelial cells are contained within said cartridge.

34. The ACS of claim 33 wherein said endothelial cells are immobilized on the outer capillary wall within said cartridge.

15 35. The ACS of claim 33 wherein said endothelial cells are immobilized on the inner capillary wall within said cartridge.

36. The ACS of claim 33 wherein said hematopoietic growth factor is selected from the group consisting of IL-1, IL-1 $\alpha$ , IL-1 $\beta$ , G-CSF, GM-CSF, IL-3, IL-6, IL-11, erythropoietin, LIF, PIXY-321 and SCF.

20 37. The ACS of claim 33 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as an enriched population of CD34<sup>+</sup> cells.

38. The ACS of claim 33 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as a subpopulation of CD34<sup>+</sup> cells contained within a mixed cell population.

39. The ACS of claim 37 wherein said CD34<sup>+</sup> cells are enriched using an antibody specific for the CD34 cell surface antigen.

40. The ACS of claim 37 wherein said CD34<sup>+</sup> cells are enriched from a source of cells selected from the group consisting of bone marrow cells, peripheral blood cells, umbilical cord blood cells.

41. The ACS of claim 38 wherein said subpopulation of CD34<sup>+</sup> cells are included within a mixed cell population selected from the group consisting of bone marrow cells, peripheral blood cells, and umbilical cord blood cells.

42. The ACS of claim 33 wherein the capillaries of said ACS cartridge are coated with a suitable coating reagent.

43. The ACS of claim 42 wherein said coating reagent is an adhesion protein having an amino acid sequence substantially identical to that shown in Figure 10 (SEQ ID NO. 1).

44. A method for the *ex vivo* expansion of hematopoietic cells comprising:

(a) coating the capillaries of an artificial capillary system (ACS) cartridge with an adhesion protein having an amino acid sequence substantially identical to that shown in Figure 10 (SEQ ID NO. 1);

(b) inoculating CD34<sup>+</sup> cells into said ACS cartridge;

(c) perfusing said ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of hematopoietic cells;

(d) culturing said CD34<sup>+</sup> cells in said ACS cartridge for a sufficient amount of time to achieve hematopoietic cell expansion.

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45. The method of claim 44 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as an enriched population of CD34<sup>+</sup> cells.

46. The method of claim 44 wherein said CD34<sup>+</sup> cells are inoculated into said ACS cartridge as a subpopulation of CD34<sup>+</sup> cells contained within a mixed cell population.

47. The method of claim 46 wherein said subpopulation of CD34<sup>+</sup> cells are contained within a population of mononuclear cells selected from the group consisting of bone marrow mononuclear cells, peripheral blood mononuclear cells, and umbilical cord blood mononuclear cells.

48. A method for transducing *ex vivo* expanding hematopoietic cells, including hematopoietic stem and progenitor cells, with packaged retrovirus vectors comprising:

(a) inoculating endothelial cells capable of supporting expansion of the hematopoietic cells into an artificial capillary system (ACS) cartridge;

(b) inoculating CD34<sup>+</sup> cells into said ACS cartridge;

(c) perfusing said ACS cartridge with culture medium containing at least one hematopoietic growth factor capable of stimulating expansion of the hematopoietic cells;

(d) culturing said CD34<sup>+</sup> cells in the presence of the packaged retrovirus vectors in said ACS cartridge for a sufficient amount of time to achieve expansion and transduction of the hematopoietic cells.

49. The method of claim 48 further comprising:

(e) harvesting cultured cells which have been transduced with the retrovirus vector from said ACS cartridge.

50. The method of claim 49 further comprising:

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(f) detecting expanded CD34<sup>+</sup> cells which are transduced with the retrovirus vector.

51. The method of claim 49 further comprising:

(f) detecting expanded CD34<sup>+</sup>CD38<sup>-</sup> hematopoietic stem cells  
5 which are transduced with the retrovirus vector.

52. The method of claim 49 further comprising:

(f) detecting expanded CFU-GM hematopoietic progenitor cells  
which are transduced with the retrovirus vector.

53. The method of claim 48 wherein said retrovirus vector contains  
10 a heterologous gene encoding a therapeutically effective product.

54. The method of claim 49 further comprising:

transplanting a therapeutically effective amount of said transduced  
hematopoietic cells to a patient.

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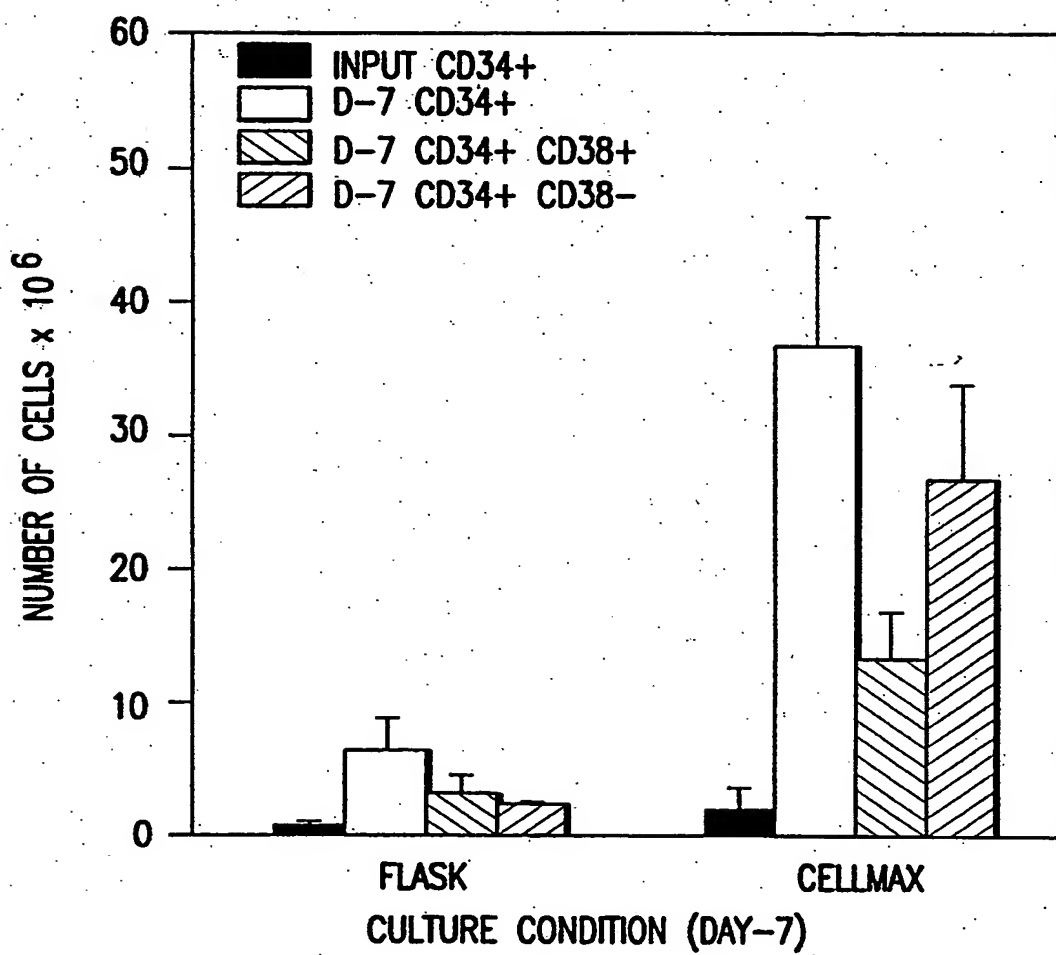


FIG.1

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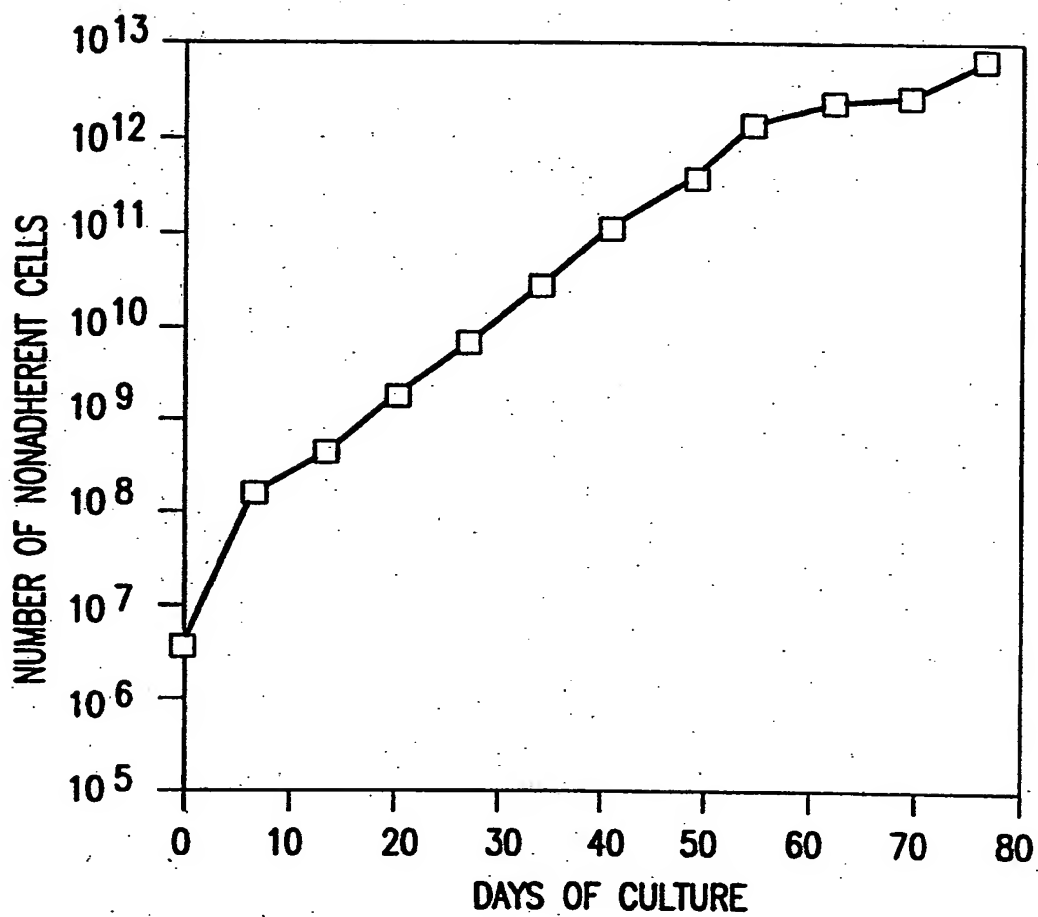


FIG.2

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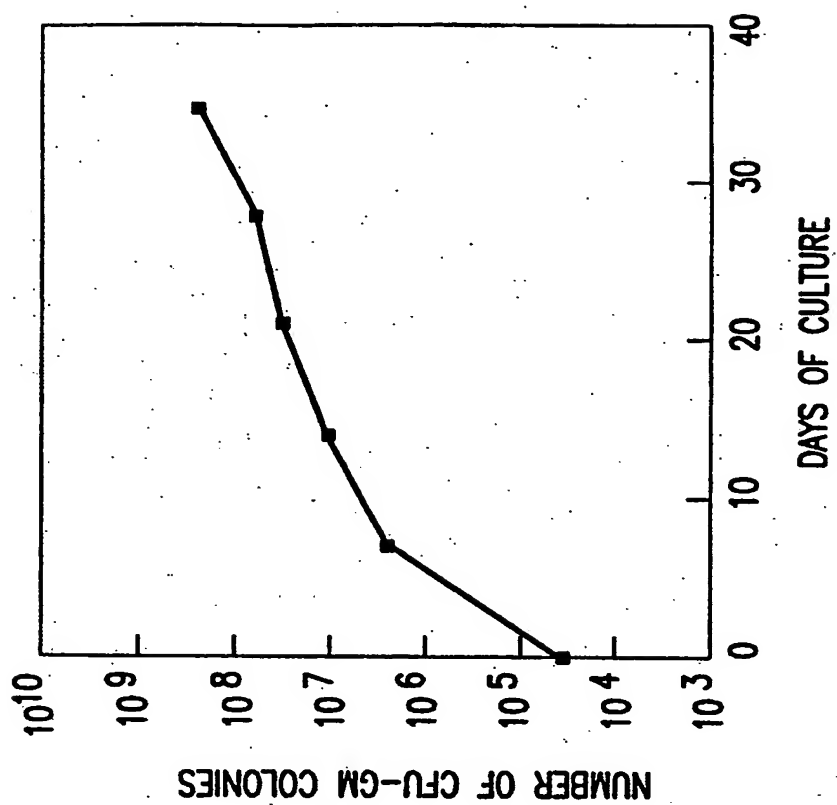


FIG. 4

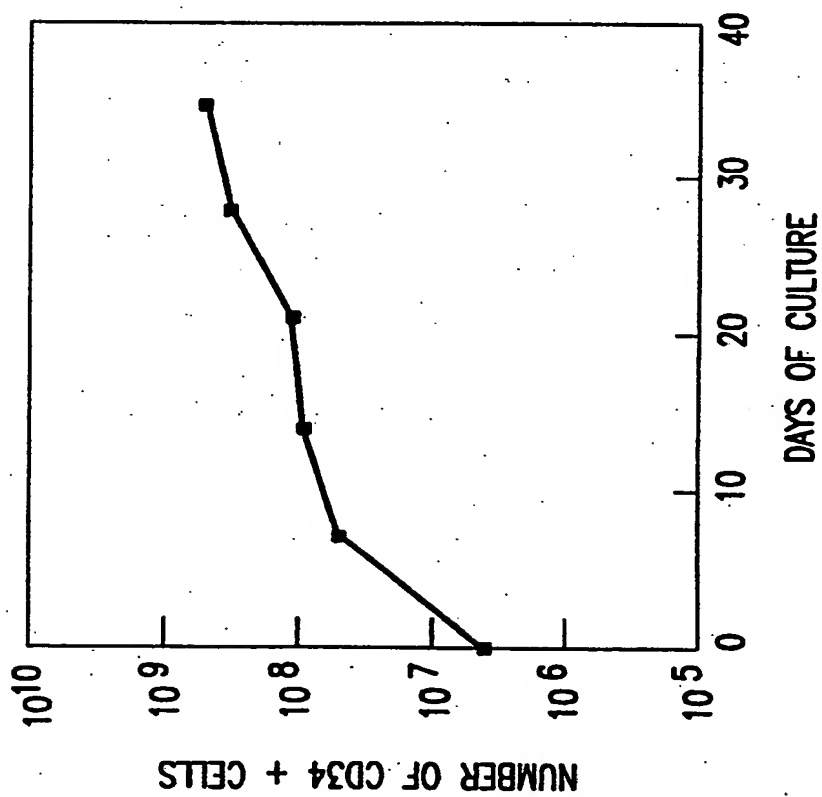


FIG. 3

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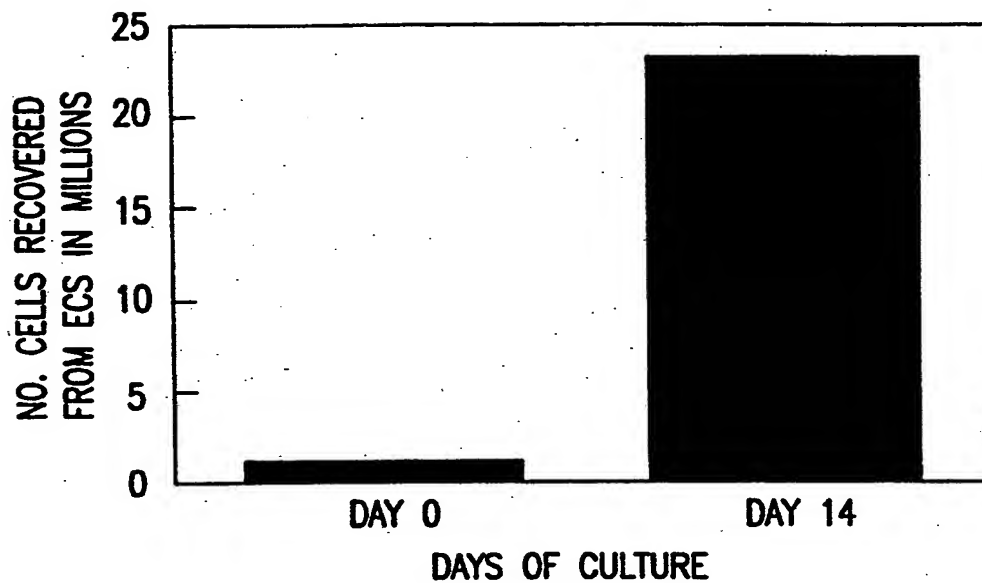


FIG.5

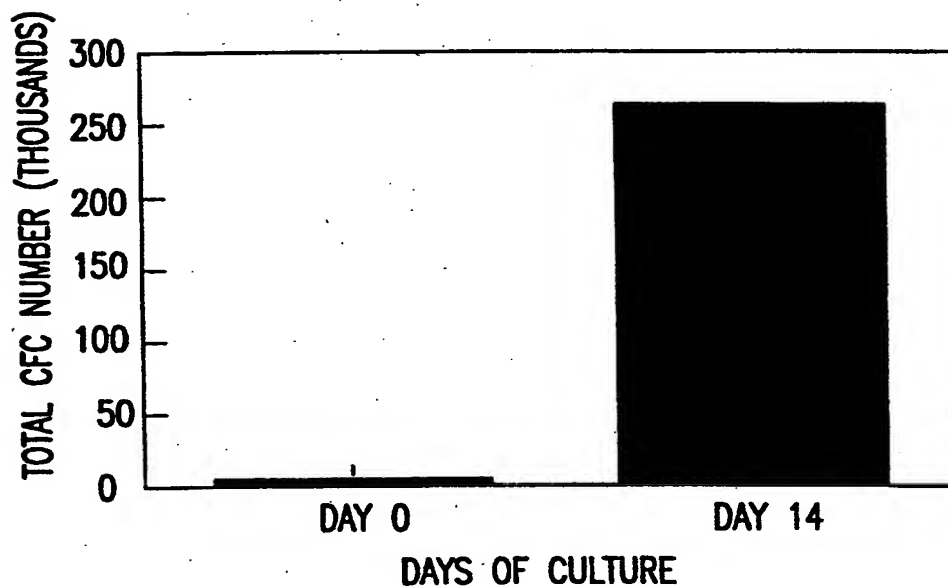


FIG.6

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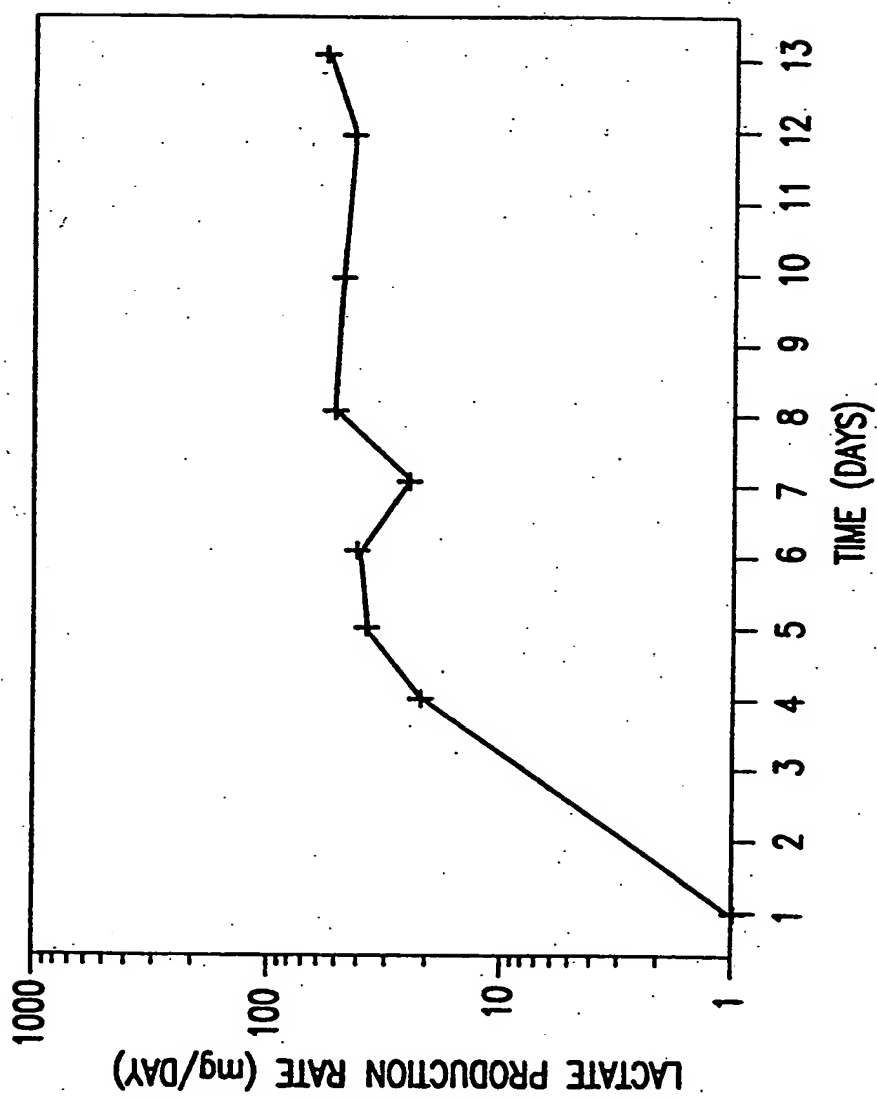


FIG. 7

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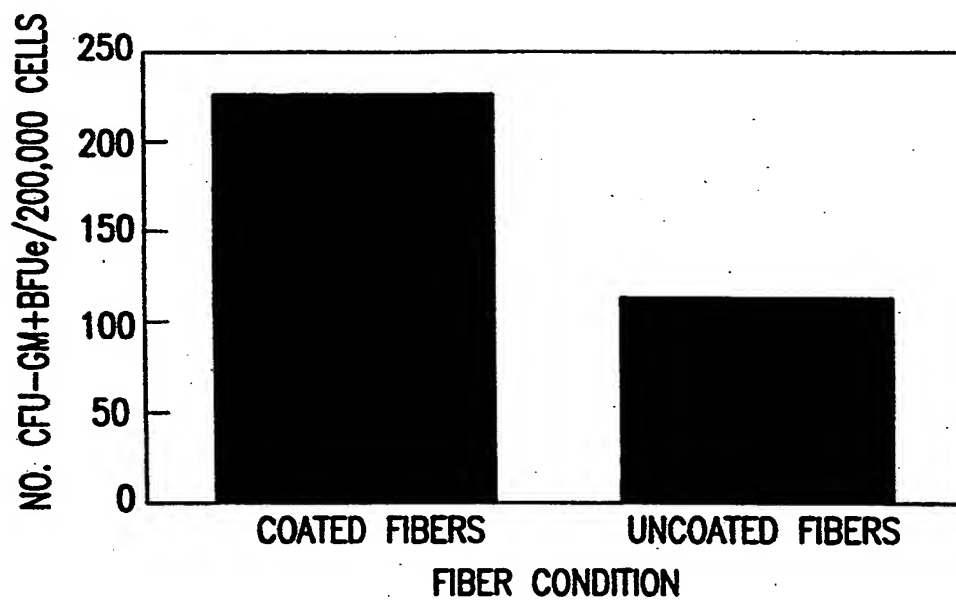


FIG.8

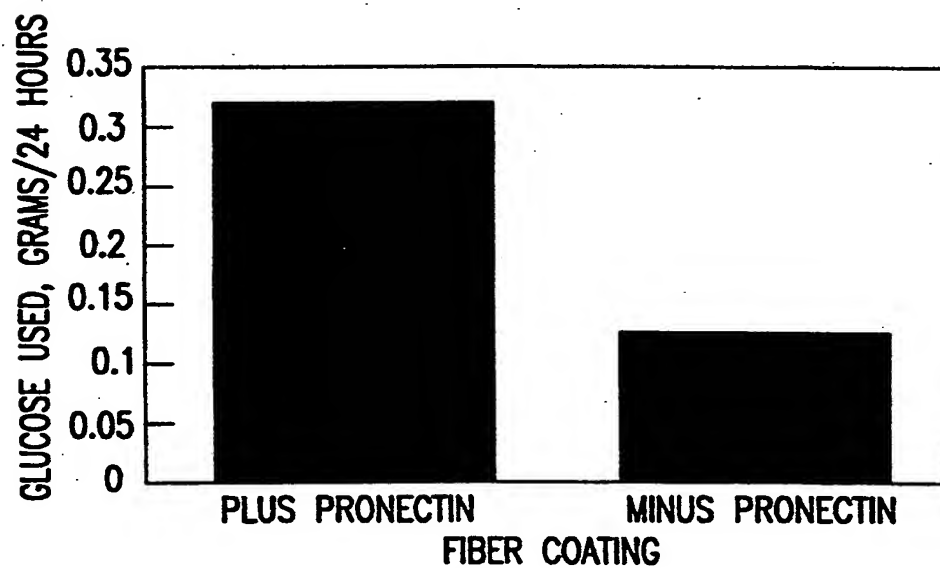


FIG.9

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Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
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 35 40 45  
 Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 50 55 60  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 65 70 75 80  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 85 90 95  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Val Thr Gly  
 100 105 110  
 Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser  
 115 120 125  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 130 135 140  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 145 150 155 160  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
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 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 195 200 205  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 210 215 220  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 225 230 235 240  
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 245 250 255  
 Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
 260 265 270

FIG.10A

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Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 275 280 285  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 290 295 300  
 Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Val  
 305 310 315 320  
 Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala  
 325 330 335  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 340 345 350  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 355 360 365  
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 420 425 430  
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 515 520 525  
 Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala  
 530 535 540

FIG. 10B

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Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
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 625 630 635 640  
 Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
 645 650 655  
 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Val Thr Gly  
 660 665 670  
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 Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
 785 790 795 800  
 Gly Ala Gly Ala Gly Ser Gly Ala Ala Val Thr Gly Arg Gly Asp Ser  
 805 810 815

FIG. 10C

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Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
                     820                    825                    830

Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
                     835                    840                    845

Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
                     850                    855                    860

Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Ala Val  
                     865                    870                    875                    880

Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala Ala Gly Gly Ala Gly Ala  
                     885                    890

Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
                     900                    905                    910

Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser  
                     915                    920                    925

Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala  
                     930                    935                    940

Gly Ser Gly Ala Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ala  
                     945                    950                    955                    960

Ala Gly Gly Ala Gly Ala Gly Ser Gly Ala Gly Ala Gly Ser Gly Ala  
                     965                    970                    975

Gly Ala Gly Ser  
                     980

FIG. 10D

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/00817

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00

US CL :424/93.21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

search terms: artificial capillary system, CD34 +, transplantation, porcine endothelial cells

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,220,725 (KNAZEK ET AL) 02 September 1980, see entire document.	1-54
Y	Biotechnology, Volume 10, issued October 1992, Edgington, "New horizons for stem-cell bioreactors", pages 1099-1104, see entire reference.	1-54
Y	Microvascular Research, Volume 37, issued 1989, Tontsch et al., "Isolation, characterization, and long-term cultivation of porcine and murine cerebral capillary endothelial cells", pages 148-161, see entire reference.	22, 23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

03 APRIL 1995

Date of mailing of the international search report

21 APR 1995

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/00817

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Blood, Volume 67, No. 2, issued February 1986, Berenson et al., "Elimination of Daudi lymphoblasts from human bone marrow using avidin-biotin immunoadsorption", pages 509-515, see entire reference.	1-10
Y	Biotechnology, Volume 11, issued March 1993, Palsson et al., "Expansion of human bone marrow progenitor cells in a high cell density continuous perfusion system", pages 368-372, see entire reference.	1-54
Y	MRS BULLETIN, issued October 1992, Cappello, "Genetic production of synthetic protein polymers", pages 48-53, see entire reference.	31, 32, 42-44
Y	Science, Volume 226, issued 26 October 1984, Anderson, "Prospects for human gene therapy", pages 401-409, see entire reference.	48-54